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ON THE KINETICS OF HÆMOLYSIS BY LYSINS OF BACTERIAL ORIGIN.—I.

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ONE of us has already shown in an extensive series of studies that the lysis produced by such simple substances as saponin, the bile-salts and the soaps may be explained in terms of a monomolecular reaction taking place between the lysin and a cell component, probably protein in nature. To this fundamental reaction, which involves a combination of the lysin with the particular component attacked, the cells offer different resistances, some being hæmolysed when only a comparatively small quantity of the component is transformed by the reaction, and others resisting lysis until a much larger quantity of the component has been transformed. The expressions which, taken together, describe the reaction in a complete manner are therefore two, one relating to the velocity of the fundamental reaction, and monomolecular in type, and the other relating to the differences in resistance on the part of the cells, and of a form similar to that applicable to curves for frequency distributions (Ponder, 1926, 1927[1]). These expressions are deduced from experimental results which are obtained by a very exact technique, by which not only the time taken for complete lysis of the cell-suspensions, but also the numbers of cells hæmolysed from moment to moment during the course of the reaction, are measured.

So far as the kinetics of the fundamental reaction between the lysin and the cell component are concerned, the differences in resistance of the cells can be ignored altogether by measuring the relation between the time taken for the lysis of an entire population of cells and the concentration of lysin acting on it. For all the simple hæmolysins, the fundamental reaction is of the same type, and its velocity is dependent on the concentration of lysin at any given moment. It was with the object of discovering whether the hæmolysins of bacterial origin are described by expressions of the same kind as those applicable to the simpler lysins of known constitution that this investigation was commenced, and this paper deals with the results obtained.

METHODS.

1. *Cultural methods.*—Except where mentioned below, all the organisms used for the production of lysins were grown aerobically in a veal-extract medium containing 0·1% of glucose and 0·2% of Na_2HPO_3, and standardized to pH 7·6. After 18 hours' incubation at 37°C. a satisfactory lysin was generally obtained. In all cases the medium was centrifuged in order to remove the organisms; filtration is inadvisable, for many hæmolysins are removed by the filter owing to their surface active properties (McLeod, 1912).

In some cases we found it difficult to obtain a satisfactory lysin by the method described, especially from *Streptococcus pyogenes*, *Staphylococcus pyogenes aureus*, and *B. coli*. In these cases, even when the lysins were obtained, they were found to be extremely labile, and to disappear within a few hours at room temperature. We have no doubt that more powerful and stable lysins can be obtained from growths in serum media, as suggested by McLeod (1912); we have, however, avoided such media, as serum is known to inhibit the action of both the simple lysins and many of the bacterial lysins, and in the case of the former at least the inhibition is of such a nature as to make quantitative work exceedingly difficult (Ponder, 1923, 1924). The use of serum media would thus introduce difficulties which we are anxious to avoid.

2. *Methods for examination of lysins.*—The methods employed were almost identical with those developed by one of us for the study of the properties of the simpler hæmolysins.

The medium containing the lysin having been freed from organisms by centrifuging, a series of dilutions is made with 0·85% NaCl. These dilutions may range from 1 in 2 to 1 in 200 or even more, depending on the activity of the lysin. Of each dilution 0·8 c.c. is placed in a series of small tubes of clear white glass ($3'' \times \frac{1}{2}''$), the tubes having been thoroughly cleaned by repeated washings and a final treatment with live steam. It is important that the tubes should be dry. To each of the series is next added 0·8 c.c. of 0·85% NaCl; the tubes are then placed in a specially constructed water-bath, warmed to 37°C., and possessing glass sides. Through these glass sides the tubes can be seen without the necessity of removing them from the water, and if a light illuminating a white ground is arranged behind the bath, the end-point which corresponds to complete lysis of the suspension can be determined with great ease.

In the water-bath is also placed a vessel containing the blood-suspension to be added to the tubes; this is prepared as follows: To four centrifuge tubes each containing saline (0·85% NaCl) is added 1 c.c. of blood, either defibrinated or oxalated, as may be more convenient. The cells are washed three times with saline, and finally suspended in 20 c.c. of saline; this forms what we term the "standard" suspension. Before use it must be suitably diluted, and for the following reasons:

As the object of the experiments is to determine, among other things, the time required for the complete lysis of the cell suspension, it is obviously of little value to compare two suspensions, as, for example, one prepared from the blood of the sheep and the other from the blood of man, when the two contain different numbers of red cells. This difficulty might be overcome by diluting

one of the suspensions in such a way as to make the two cell-contents equal, but even this procedure is inadequate, for the " zone of action" for the lysin may be different in the case of one cell suspension from what it is in the case of the other. The idea of this " zone of action " may be found developed in two earlier papers (Ponder, 1926, 1927); briefly, it is necessary in order that we shall take account of the fact that the lysin can act at the region of the surface of the cells, and there only. A portion of the fluid round each cell accordingly contains all the molecules of lysin which have the opportunity of entering into the hæmolytic reaction, and this portion of fluid we call the zone of action. Molecules outside the zones cannot enter into the reaction at all. Now, in a concentrated cell suspension many of the zones overlap, for they are of considerable size, having radii of the order of 5μ or more. Lysis in such a suspension cannot proceed in a way comparable to lysis in a suspension in which the zones are all separated, principally because when any two zones overlap, the lysis of one of the cells intereferes with the lysis of the other, whereas, when the zones are all separate, each cell with its surrounding zone constitutes a little hæmolytic system in itself, uninfluenced by neighbouring reactions. While the theory of the matter will be found in the papers mentioned above, the practical point for our present investigation is that we shall work with suspensions in which the zones are completely separated, in order that all suspensions, prepared from whatever source, may be comparable.

The standard cell suspension, prepared as above, must therefore be diluted with saline until it is as dilute as is possible to work with. The exact degree of dilution depends, of course, on the number and density, as regards light-stopping power, of the cells in the suspension as originally prepared; dilutions of from 1 in 10 to 1 in 20 are usually adequate. The resulting suspension is very pale, but its complete lysis is quite readily determined; we may, moreover, be certain that the dilution is such as to separate the cells with their surrounding zones of action as much as is consistent with practicability, for any greater degree of separation would depend on the suspension being so diluted as to make the determination of the end-points impossible.

The suspension and the series of dilutions of lysin having acquired the temperature of the water-bath, 0·4 c.c. of suspension is added with a warmed pipette to each tube containing lysin. The moment of addition of the suspension and the moment of complete lysis are noted with a stop-watch; the lysis of the cells is thus allowed to proceed without the removal of the tubes from the bath. The time required for complete lysis of the suspension by any dilution of lysin is therefore known exactly; small errors only should occur, as the end-point can be determined with great accuracy.

The dilution of lysin is then plotted, expressed as an arbitrary concentration, against the time taken by it for the completion of lysis of the quantity of suspension used; in this way a curve, such as shown in Fig. 1, is obtained. This curve is then fitted with the expression—

$$\kappa t = \log \frac{c}{c-x} \quad . \quad . \quad . \quad . \quad . \quad (1)$$

in which t is the time in minutes required for the completion of lysis, c the initial concentration of lysin in the system, measured in some arbitrary units,

x the amount of lysin used up in producing lysis, measured in the same units as c, and κ a constant.

To find the value of x, we find the asymptote of the curve where $t = \infty$ and $c = x$. This is found first by inspection, and afterwards by trial; the value of c corresponding to the asymptote gives the value of x. The constant κ is then found from the expression given above.

RESULTS.

1. *B. welchii.*—We have obtained satisfactory lysins from phosphate broth cultures by allowing growth to take place anaerobically under liquid paraffin for 18 hours at 37°C. Under these circumstances a powerful lysin results; satisfactory lysins have also been obtained from cultures incubated for 2 days.

With a suspension of sheep cells so diluted as to separate the zones of action as much as possible, the following relations between t, the time required for complete lysis of the suspension, and c, the concentration of lysin acting on the cells in the system, was obtained at 37°C. The units in which c is measured are necessarily arbitrary, for the concentration of lysin in the undiluted medium is unknown; the concentration of lysin present in the first tube of the series of dilutions was therefore taken as unity:

c.	t, experimental.	t, calculated.
0·05	0·6	0·47
0·02	1·3	1·20
0·01	3·0	2·82
0·005	9·0	9·00
0·0045	13·1	12·28

The values of t were calculated from the values $x = 0{\cdot}004$ and $\kappa = 0{\cdot}178$. The curve obtained by the plotting of the above values may be compared, as regards general form, with a similarly obtained curve for the hæmolysin saponin (Figs. 1 and 2). Both curves are described by expression (1) with a

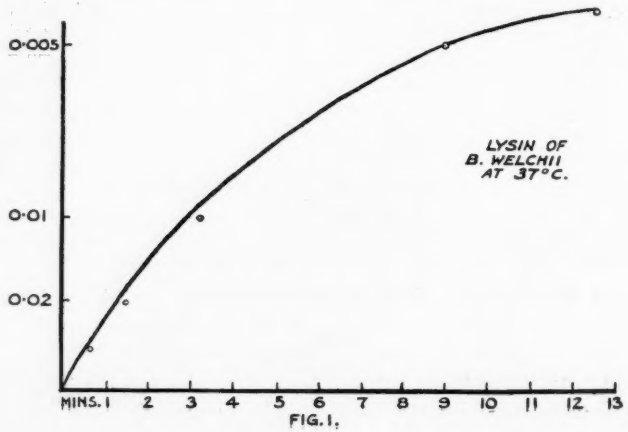

FIG. 1.

high degree of accuracy; it may therefore be concluded that, so far as the time concentration curves at least are concerned, the lysin produced by *B. welchii* is similar to a simple lysin such as saponin, one of the glucosides, one of the bile-salts, or one of the soaps.

This being so, we may treat the lysin of *B. welchii* as a simple hæmolysin, and may proceed to find the differences in resistance to its action on different types of red cell. The essential point is that all the suspensions to be compared shall be comparable as regards the state of separation of the cells; this, it has already been explained, is ensured by suitable and sufficient dilution.

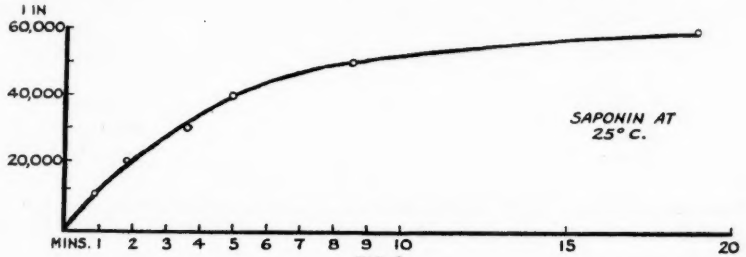

FIG. 2.

The following table shows the results for five different suspensions, obtained from the blood of the sheep, the ox, the horse, the rabbit, and man. Experimental results alone are given:

c.	*t*, sheep.	*t*, ox.	*t*, horse.	*t*, rabbit.	*t*, man.
0·05	0·6	0·5	0·65	0·65	0·7
0·02	1·3	1·1	1·4	1·4	1·6
0·01	3·0	2·6	3·3	3·2	3·9
0·0055	—	—	—	—	13·5
0·0050	9·0	8·0	13·0	13·0	—

The best values for x, taking κ as 0·178 throughout, will be found to be—

x, ox	0·0038
x, sheep	0·0040
x, horse	0·0045
x, rabbit	0·0045
x, man	0·0050

These values fit the experimental results excellently. From them we see that there are only very small differences in resistance between the cells of the different species, but that the order of resistance is as follows, beginning with the least resistant: ox, sheep, rabbit and horse, man.

To express the relative resistance as a figure, we use the value of the ratio—

$$\frac{x_2}{x_1} = R \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

where x_2 is the amount of lysin used up in hæmolysing the suspension whose resistance is to be found, and where x_1 is the amount required for the lysis of

a suspension which is selected as a standard. The advantage of this method of measuring the relative resistance is that we obtain the same result if we take the ratio—

$$\frac{c_2}{c_1} = R \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

in which c_2 and c_1 are any two concentrations of lysin which take the same time to bring about lysis, when acting on the suspension whose resistance is to be determined and on the standard suspension respectively.

In the case of the suspensions examined above, we have in this way the following values of R, the resistance constant, referred to the resistance of the suspension of sheep cells as the standard and as unity:

R, ox	0·95
R, sheep	1·0
R, horse	1·12
R, rabbit	1·12
R, man	1·25

The differences of resistance are far smaller than in the case of most of the simple lysins; for saponin, for instance, the values of R range from about 0·5 in the case of the rabbit to about 7·0 in the case of the sheep. The lysin of *B. welchii*, on the other hand, seems to attack the cells of all the species examined to much the same extent, and the order of resistance is quite different from that of resistance to saponin, which runs: sheep, ox, horse, man, rabbit—the least resistant species occurring last. This result with the lysin of *B. welchii* agrees in general with that obtained by Henry (1922), who found little difference between the resistance of the cells of man, the guinea-pig, the rabbit and the sheep. The cells of the horse he found to be much more resistant than those of the other species; as his observations were made with 6% suspensions of unequal cell content, much importance cannot be placed on his findings.

Regarding the nature of the lysin we are not in a position to express an opinion. Some of the earlier observers, as for instance McCampbell (1909), believed that the lytic action of *B. welchii* is due to the formation of butyric acid; the work of Bull, however, has shown conclusively that the bacillus produces a true hæmolysin (Bull, 1917). The hæmolysin is stated to be relatively thermostable, for an exposure to 60° C. for 30 minutes does not completely destroy it (Henry, 1922). The lysin is inactivated by small quantities of serum, and in this respect it resembles the simple hæmolysins such as saponin. The affinity of hæmolysins for the serum proteins, by the union with which an inert substance is produced, may be taken as presumptive evidence that such lysins act on the cells by a similar combination with their protein components.

2. *B. subtilis.*—*B. subtilis* resembles many of the members of the group of organisms to which it belongs in its power of producing a potent lysin. Subtilolysin has been investigated by Heyrovsky and Landsteiner (1907); it has been stated that the lysin is comparatively thermostable, resisting temperatures up to 56° C.

We were able to obtain, after 18 hours' growth in phosphate broth at 37° C., a good lysin, which gave the following relations between t and c for sheep cells at 37° C.:

c.	t, experimental.	t, calculated.
0·02	1·0	0·91
0·01	2·0	1·94
0·005	4·5	4·35
0·003	8·0	7·97
0·0025	14·0	14·00

The calculated results, which fit the observed values almost perfectly, were obtained from the values $x = 0{\cdot}002$ and $\kappa = 0{\cdot}115$.

The resistance constants for the different species of cells work out as the following:

R, rabbit	0·7
R, man	0·9
R, ox	0·95
R, horse	1·0
R, sheep	1·0

Again the differences are small, and the order of resistance differs from both that for saponin and for the lysin of *B. welchii*. It cannot be too strongly emphasized, however, that the order of resistance, as well as the values for the resistance constants for the cell suspensions employed, is largely dependent on the concentration of the suspensions. A concentrated suspension of the least resistant cells may easily take longer to hæmolyse than a more dilute suspension of much more resistant cells, for no other reason than that there are more cells to hæmolyse. This point seems to be persistently overlooked, and we too frequently find comparisons between, say, a 1% suspension of sheep cells and a 1% suspension of human cells, irrespective of the fact that the first suspension contains twice as many cells as does the second. When great differences between the resistance of the cells exist, as in the case of the resistance of these two species to saponin, the error matters little, but if the differences of resistance are small, the inequality of cell content may easily reverse the correct order of resistance.

3. *B. tetani*.—Ehrlich (1898), who first recognized the hæmolysin in filtrates of cultures of *B. tetani*, applied the term "tetanolysin" to the hæmolytic agent in the toxin of this organism. The properties of tetanolysin were studied by Madsen (1899) by quantitative methods; his wholly correct conclusion, that the lysin acts in a manner described by a monomolecular expression, is without support from his own experiments. Brooks (1921) and one of us (Ponder, 1926) have pointed out the errors by which this conclusion was arrived at; it happens that an error in theoretical treatment cancelled out with an error due to faulty experiment, and that thus the correct result appeared by a process of elimination.

Grown in phosphate broth anaerobically, the bacillus gives after 2 to 3 days, in our experience, a lysin of moderate activity. To obtain a more active lysin, we grew the bacillus in Robertson's bullock heart medium for 2 days.

A powerful lysin resulted, and the following relations between t and c were obtained for sheep's cells at 37° C.:

c.	t, experimental.	t, calculated.
0·1	0·8	0·74
0·05	1·8	1·68
0·03	2·8	2·80
0·025	4·0	4·16
0·020	6·4	6·26

The calculated results were obtained from the values $x=0{\cdot}0143$ and $\kappa=0{\cdot}2$. The correspondence between calculated and observed results is excellent.

Turning to the question of the relative resistances of different species of cells, we have found the following resistance constants:

R, rabbit	0·90
R, ox	0·97
R, horse	0·97
R, man	0·97
R, sheep	1·0

Kraus and Clairmont (1900) found little difference in resistance between the cells of the rabbit, ox and horse; they note that the cells of the goat and pig are more resistant than those of the animals mentioned.

4. *B. anthracoides.*—This member of group of the aerobic, Gram-positive, sporing bacilli differs from *B. anthracis* in producing a powerful lysin as well as in being of slighter pathogenicity. The lysin has been investigated by Jarmai (1913), Wagner (1923), and Hallermann (1925).

We have found that young cultures give the better lysins in phosphate broth, an excellent hæmolysin being produced after growth for 18 hours in that medium. The relation between c and t for sheep cells at 37° C. is shown in the following table:

c.	t, experimental.	t, calculated.
0·05	1·1	1·14
0·025	2·5	2·70
0·020	4·0	3·92
0·016	5·5	5·54
0·0143	11·5	10·87

The values were calculated from $x=0{\cdot}0125$ and $\kappa=0{\cdot}25$. The resistance constants were found to be the following:

R, man	0·85
R, rabbit	0·90
R, ox	0·90
R, horse	1·0
R, sheep	1·0

These differences are so small that it is very difficult to ascertain the exact order in which the different types of cell should be placed.

5. *El Tor vibrio.*—The production of lysin by this organism, which was

first isolated by Gottschlich in 1905, is the chief feature distinguishing it from the classical cholera vibrio. The lysin has been studied by Kraus and Přibram (1906); it is demonstrable in broth after 2 days' incubation, and is destroyed on heating to 58° C. We have found it a little more difficult to obtain than the lysins already considered, but we were able to produce satisfactory lysins by aerobic growth in phosphate broth for periods of from 1 to 2 days at 37° C.

The relations between c and t are shown in the following table, which refers to sheep cells at 37°C. The calculated values were obtained from $x=0{\cdot}0909$ and $\kappa=0{\cdot}48$. The value of κ is surprisingly large. It will be observed that the lysin is not a very active one, producing lysis in dilutions of about 1 in 10 only; this appears characteristic of lysins obtained from phosphate broth cultures of the organism, for we have scarcely ever found a lysin which would bring about lysis in dilutions of more than 1 in 20.

c.	t, experimental.	t, calculated.
0·5	0·5	0·41
0·25	1·0	0·94
0·20	1·3	1·28
0·125	2·9	2·69
0·100	5·0	5·00
0·095	7·0	6·43

The relative resistances of the various types of cells are shown by the following values of R:

R, rabbit	0·8
R, sheep	1·0
R, horse	1·0
R, ox	1·1
R, man	1·3

These differences are quite small, although a little larger than in the case of most of the other bacterial hæmolysins.

As stated by Kraus and Přibram, the lysin of El Tor vibrio is inactivated by heating to 58°C. for about 30 minutes. It is also inhibited by small quantities of serum (0·1 c.c. of 1 in 20 rabbit or horse-serum). In the latter and former respects it is similar to most other bacterial hæmolysins, and in the latter respect to the simple hæmolysins.

6. *Streptococcus pyogenes.*—The production of lysins in culture media containing streptococci was investigated by Besredka in 1901, and has since been extensively studied. The lysin is rapidly produced, and attains a maximum in from 7 to 18 hours, according to the composition of the culture medium. It is stated by von Hellens (1913) that the most favourable medium for the production of streptolysin is serum broth containing 40 to 50% of horse-serum. This medium we have been unable to use, for the reason stated above. The addition of sugar, especially of glucose, is said to interfere with the production of the lysin (Lyall, 1914).

We have obtained lysins of no great activity in the phosphate broth medium after from 18 to 24 hours' growth at 37°C. The lysins thus produced we found to be extremely labile, often disappearing within a few hours at room

temperature. The relations which we obtained between c and t, for sheep cells at 37°C., are set out in the following table:

c.	t, experimental.	t, calculated.
1·0	1·0	1·10
0·5	2·3	2·47
0·3̇	4·0	4·29
0·25	6·5	6·87
0·20	11·0	11·00

The values were calculated from $x=0{\cdot}16\dot{}$ and $\kappa=0{\cdot}1613$. The following resistance constants were obtained:

R, horse	0·95
R, sheep	1·0
R, man	1·1
R, rabbit	1·3
R, ox	1·6

This order does not agree with that given by v. Hellens (1913), who places the different types of cell in the following order, the least resistant occurring first: rabbit, dog and guinea-pig, man, horse and ox, sheep and goat. The suspensions by the examination of which this order was determined by him were, however, not comparable as regards cell content, and it is significant that the suspensions which were found to be of the highest resistance are those from animals which have a high red-cell count, while the least resistant suspensions include that of the cells of the rabbit, an animal from whose blood notoriously weak and unsatisfactory suspensions result.

The hæmolysin is inactivated by heating to 60°C. for 1 hour, and is inhibited by small quantities of serum. It appears to be a substance which possesses considerable surface activity, for it is readily adsorbed on filters (McLeod, 1912; v. Hellens, 1913).

7. *Staphylococcus pyogenes aureus.*—Since the investigations of Kraus and Clairmont (1901), and of Neisser and Wechsberg (1901), the hæmolytic properties of *Staphylococcus pyogenes* have been accepted as an important characteristic of that organism. In broth cultures a hæmolysin appears about the 6th day, reaches its maximum about the 9th day, and disappears between the 13th and 19th days. The lysin is also developed on an agar slope, and can be obtained by washing the growth with saline (Oppenheimer, 1911). It is of interest to note that the hæmolysin is probably associated with proteolytic substances, as observed by Julianelle (1922).

In phosphate broth we were able to obtain only weak lysins. The relations between t and c were found to be as follows for sheep cells at 37° C.:

c.	t, experimental.	t, calculated.
1·0	1·6	1·45
0·5	3·7	3·41
0·3̇	7·0	6·66
0·25	14·0	14·00

The calculated values were taken from $x=0{\cdot}23$ and $\kappa=0{\cdot}18$.

The resistance constants for this lysin acting on different types of cells was found to be the following:

R, horse	0·85
R, ox	0·85
R, rabbit	0·90
R, sheep	1·0
R, man	1·1

As in the case of the other bacterial hæmolysins examined, these differences are very small.

8. *B. histolyticus.*—We have found that this organism produces quite a powerful lysin when grown anaerobically in phosphate broth for a period of from 18 to 24 hours at 37° C. The lysin is of particular interest, since *B. histolyticus* is a proteolytic organism.

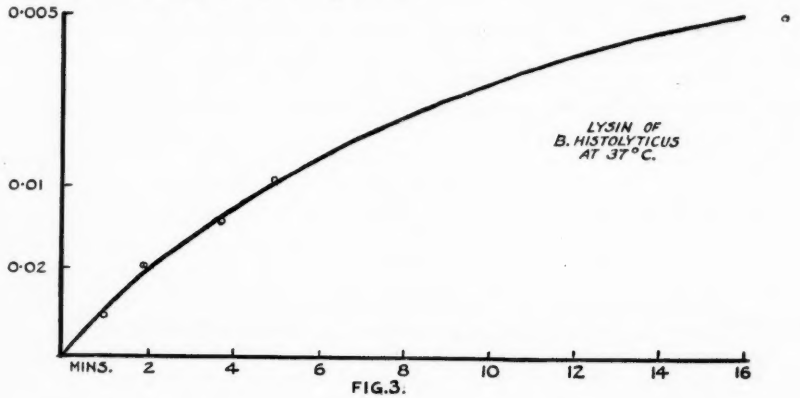

FIG. 3.

The relations between c and t, for sheep cells at 37° C., are as follows:

c.	t, experimental.	t, calculated.
0·05	1·0	0·85
0·02	2·0	2·15
0·0125	3·9	3·85
0·01	5·0	5·07
0·005	17·0	16·09

The calculated values are taken from the values $x = 0{\cdot}004$ and $\kappa = 0{\cdot}1$. The relation between the variables is shown graphically in Fig. 3.

The resistance of cells of different species was found to be as follows:

R, sheep	1·0
R, ox	1·05
R, rabbit	1·1
R, man	1·5
R, horse	1·7

Again the differences in resistance are very small, but it will be noticed that the order in which the species fall as regards resistance is quite different from the orders obtained with other bacterial lysins.

In view of the many statements made regarding the mode of action of these lysins and of the simpler lysins such as saponin, it is of interest to find the lysin of *B. histolyticus*, an organism which attacks proteins, presenting the same features as those presented by saponin, the bile-salts and the soaps. We are inclined to regard this as additional evidence in support of the hypothesis advanced by one of us, that the simpler lysins act by attacking the protein component of the red cell.

Like saponin and the other simple lysins, the lysin of *B. histolyticus* is inhibited by serum or plasma in high dilutions. It is also inactivated by heating to 56° C. for 30 minutes.

9. *B. coli.*—The occurrence of hæmolytic strains of *B. coli* has been described by Kayser (1903), Dudgeon, Wordley and Bawtree (1921), and others. The lysin is remarkable in that it is not altered by an exposure to 120° C. for 30 minutes (Kayser, 1903). The hæmolysins which we have been able to obtain in phosphate broth have been extremely poor and very unstable; in our experience 18-hour growths in this medium yield as good a lysin as we have been able to prepare.

The relations between t and c, for sheep cells at 37° C., are set out below:

c.	t, experimental.	t, calculated.
1·0	2·5	2·80
0·5	6·5	7·28
0·3	20·0	18·40

The calculated results are obtained from the values $x = 0{\cdot}3$ and $\kappa = 0{\cdot}125$. As will be seen from the figures, the activity of the lysin is very small.

Investigation into the relative resistance of cells of different species to this lysin showed no difference in the resistance of the cells of the sheep, ox, horse, rabbit, and man. The failure to arrange these in a resistance series may in part be due to the inactivity and instability of the lysin; even so, the differences must be very small.

CONCLUSIONS.

1. So far as the fundamental nature of the reaction is concerned, there is evidence to show that the hæmolysins of bacterial origin are very similar to the hæmolysins of the saponin class. In both cases, under the special conditions of these experiments, the expression applicable to monomolecular reactions describes the results in an adequate way. It is important that this similarity should have been established, for it enables us to group the bacterial lysins, for the purposes of quantitative treatment, among the simple hæmolysins, and to treat them in a similar manner.

2. There is evidence to show that most, if not all, of the bacterial lysins unite with protein, whether in plasma, in serum, or in the red-cell envelopes. In this respect, also, they resemble the simple hæmolysins. We cannot, however, suggest that the mode of action of these two groups of lysins bears more than a superficial resemblance. Here it may be mentioned that the

lytic effects observed in these experiments are not directly dependent on such factors as pH or tonicity, for the dilution with normal saline in which the lysins act, as well as the pH and tonicity of the culture medium, make such an explanation of their action impossible.

3. The bacterial lysins differ from the simpler lysins in that the cells of different species offer to their action very slight differences of resistance. If the cells of the various species are arranged according to their resistance, the series will be found to be different for almost every bacterial lysin. There is thus no correlation between protein content and resistance to the bacterial lysins, such as certainly exists in the case of saponin (Ponder, 1927[2]). This does not, of course, preclude the action of the lysin being upon a protein component.

SUMMARY.

1. So far as the kinetics of the fundamental reaction are concerned, the hæmolytic action of the lysins of bacterial origin is identical with that of saponin and the simple hæmolysins.

2. The cells of different species offer to the action of the bacterial hæmolysins very much the same resistance, such differences as can be found being exceedingly small.

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ON THE NATURE OF THE HYPERGLYCÆMIC RESPONSE TO INJECTIONS OF CERTAIN KILLED BACTERIA.

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THE relation of infection to hyperglycæmia is a problem of especial interest in connection with the production of diabetes, and with the change in the response of a diabetic patient to insulin during an intercurrent infection.

Clinical evidence of the inefficacy of insulin in diabetic patients suffering from acute infections has been given by many, and Lawrence and Buckley (1927) have recently reviewed the literature on this subject. It is a common clinical opinion that this altered response to insulin is due to a temporary relapse of the already impaired pancreatic function. If this were so the pancreatic function would not be expected to improve rapidly after cessation of the infection, and yet the quick recovery of the normal response to insulin after the infection is removed is as striking a feature of the phenomenon as the resistance while the infection persists. Lawrence and Buckley have suggested that this resistance may be due to change in the activity of the thyroid and suprarenals during infection, and have offered, as evidence of this, experiments showing the change in reaction to insulin in rabbits poisoned by diphtheria toxin, in which case it is known that the thyroids and suprarenals are particularly affected by the toxin. The present experiments offer evidence that bacterial hyperglycæmia in the rabbit is dependent upon increased activity of the suprarenals, from which it may be deduced that altered response to insulin in infection may be related to excessive activity of the suprarenals.

That carbohydrate metabolism is greatly disturbed in man, even in non-diabetics, during acute infections is evidenced by recent reports of lowered glucose tolerance and of fasting blood-sugar values above the normal range in a variety of infections. While clinical evidence is suggestive, experimental proofs of a relationship between carbohydrate metabolism and infection have only recently been obtained. Thomas (1924) and Menten and Manning (1924, 1925, 1926) found microscopic changes in the islands of Langerhans in spontaneous *B. enteritidis* infections in guinea-pigs and rabbits, and the latter authors reported definite hyperglycæmia in a group of these spontaneously infected animals. They were unable to produce a transient hyperglycæmia by intravenous injection into normal animals of killed organisms isolated from these infected animals. There were accompanying microscopic changes in liver, islets, suprarenals and kidneys, and Menten postulated that the hyperglycæmia may be due to direct action on the liver-cell.

Zeckwer and Goodell (1925) found a similar hyperglycæmia resulting from intravenous injections of killed organisms, normal inhabitants of the gastro-intestinal tract, notably *B. coli* and *B. proteus vulgaris*. These findings have

been corroborated by Lawrence and Buckley (1927), Levine and Kolars (1926), who extended the list of bacteria eliciting this response.

Geiger (1925, 1927), in studying the effect of external temperature changes and of altered body temperature on the blood-sugar of rabbits, used "colitoxin" as a fever-producing agent, and found a hyperglycæmia which he calls "Entfieberungsglykämie," and which he considers is essentially an "Unterkühlungshyperglykämie," as the increased body temperature in contrast to the relatively lower external temperature results ultimately in chilling. He seems to assume that "colitoxin" is not specific in its action, that milk is just as effective, the essential thing being a fever-producing agent, although the blood-sugar curves do not run parallel to temperature. In his earlier experiments the rise in blood-sugar reached a maximum between the fourth and fifth hours after injection. In his later reports the maximum was reached in 1 to 2 hours' time. Splanchnicotomy prevented this fever hyperglycæmia and resulted in hypoglycæmia instead—an effect similar to that resulting when atropine was used instead of colitoxin.

Barber (1925) has tried to produce permanent hyperglycæmia by infecting the gall-bladders of dogs with fæcal material, but the resultant hyperglycæmia would seem to be dependent on gross destruction of pancreatic tissue.

Quantitative data were previously given of the hyperglycæmic response to killed bacteria (Zeckwer and Goodell, 1925). After a delay of about 30 minutes the blood-sugar rises to a maximum at about 1 to 2 hours after injection, reaching a level of 0·15% to 0·25%. The present investigation has been carried out with the object of determining the mechanism by which this bacterial hyperglycæmia is produced.

EXPERIMENTAL PROCEDURE.

Rabbits which had fasted for at least 16 hours before each experiment were used. Cats were tried, but were unsatisfactory. Blood sugar determinations were made by the Hagedorn and Jensen method on 0·1 c.c. of blood obtained from the ear veins immediately before injecting the bacterial suspension, and at frequent intervals thereafter, for a period of 4 to 6 hours. Cultures of *B. coli* and *B. proteus vulgaris* were grown on sugar-free agar slants for 24 hours, the growth washed off in 0·9% NaCl solution and killed by 60°C. heat in a water-bath for 1 hour. Bacterial counts were made by comparison with opacity tubes. The doses given varied from 2000 million to 4500 million bacteria per kgm. body-weight, as there is considerable individual variation in the response of the rabbits.

Organisms grown for a considerable time on artificial media free of sugar are more toxic to the animals and less effective in raising blood-sugar than organisms recently isolated. Where repeated injections were given to the same animals, the injections were timed so as to eliminate the possibility of anaphylactic reaction. If two experiments were more than ten days apart, a desensitizing dose was given subcutaneously at weekly intervals.

In such of the experiments as involved operative procedures immediately before the injection of the bacterial preparation, amytal was used as an anæsthetic, without any evident modification of the blood-sugar response. The

arterial blood-pressure was taken in two of these experiments, but showed no alteration in consequence of the injection.

EXPERIMENTS.

(a) *The Effect of Removing the Influence of the Suprarenals.*

The left suprarenal gland was removed and the right denervated aseptically under C.E. anæsthesia, in seven rabbits. About a week after recovery from the operation the blood-sugar concentration following injection of bacteria was compared with the curve previously obtained with the same dose in the same animals before operation.

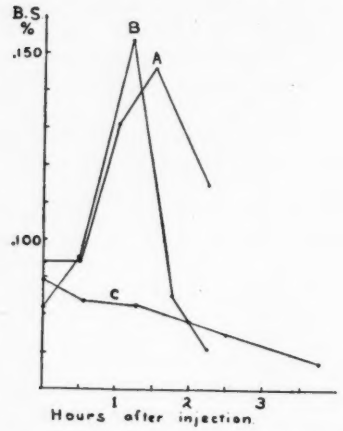

Fig 1. Response to intravenous injection of killed B.proteus R22.
A. Jan 8. Before operation
B. Jan 19 Before operation
C. Jan 28. 6 days after operation

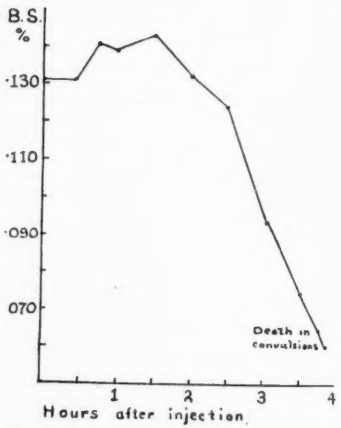

Fig. 2. Response to B.coli in Rabbit 3, after removing one suprarenal and denervating the other.

Three effects of suprarenalectomy were noted:

(1) There was complete inhibition of the rise in blood-sugar in all animals (see Table I and Fig. 1).

(2) In three cases the blood-sugar fell to a convulsive level, resulting in death (see R 3, R 5, R 47 in Table I and Fig. 2).

(3) In two cases, after a distinct fall, there occurred a slight rise approaching the preliminary level, followed by a second fall (*R* 10 and *R* 11 in Table I and Fig. 3).

(b) *The Effect of Thyroidectomy and Thyroid Feeding.*

Although the literature is extensive on the relation of the thyroid to carbohydrate metabolism, the evidence has not been altogether satisfactory. Cramer (1926) has presented evidence of histological changes in thyroid and suprarenals in infections, but such changes have not been correlated with chemical changes in the blood. Burn and Marks (1925) have shown that

thyroidectomy in rabbits results in a decreased response to adrenalin and an increased response to insulin, while thyroid feeding results first in an increased response to adrenalin and a decreased response to insulin, but later after thyroid feeding has depleted the glycogen store of the liver, the injection of adrenalin no longer results in hyperglycæmia, but may instead cause a fatal hypoglycæmia.

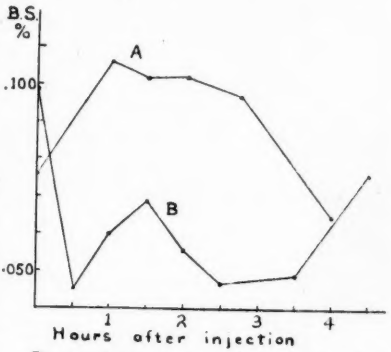

Fig. 3. Response to Killed B. coli. R11
A = Nov 25 Before operation
B = Dec 10. After operation

Since the present experiments have shown that bacterial hyperglycæmia is incident to a discharge of adrenalin from the suprarenals, it was thought that findings similar to those of Burn and Marks might be obtained with bacterial injections after thyroidectomy and thyroid feeding. The results, however, were not clear-cut. It will be seen from Table II that although there was decreased response after thyroidectomy, yet the changes did not differ very greatly from the variations which may occur in the normal control animals which were subjected to repeated injections of bacteria alone. The difficulties are that repeated injections of bacteria cause loss of weight with probable depletion of the glycogen stores, which may result in a lessened response to bacteria after several injections, as in the control rabbit R 38. Furthermore, the development of active immunity against the bacteria introduces a factor which may play a *rôle* in the reaction, although against this is the evidence, which will be given later, that the agent responsible for the hyperglycæmia is non-protein. The greatest difficulty was found in knowing at just what stage of thyroid feeding one might expect to get increased response, because when thyroid feeding is carried beyond a certain point, there is, as Burn and Marks have shown, no longer sufficient glycogen reserve to permit the occurrence of hyperglycæmia. In Table II, R 43 and R 32 had evidently been carried beyond this point. However, R 44 and R 23 showed a definite increased response after thyroid feeding—an effect which has no parallel in the controls (see Fig. 4).

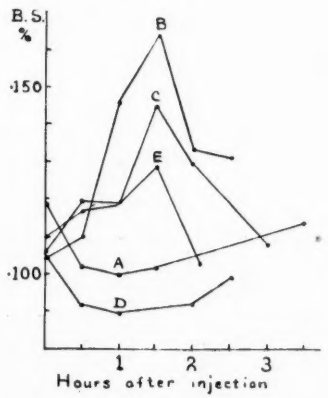

Fig. 4. Response to B. proteus after thyroidectomy and after thyroid feeding. R44
A: March 28, 14 days after thyroidectomy.
B: April 5, after thyroid feeding
C: April 22, after thyroid feeding had been stopped
D: May 7, 33 days after stopping thyroid feeding.
E: May 17, after resuming thyroid feeding

TABLE I.—*Blood-Sugar Curves following the Injection of Killed Bacteria in Normal and in Suprarenalectomized Rabbits.*

		Date of injection.	Percentage glucose in blood.								Maximum increase in blood-sugar above preliminary level.	Maximum fall in blood-sugar below preliminary level.
			Before injection.	Hours after injection.								
				½+.	1+.	1½+.	2+.	2½+.	3+.	4+.	6+.	
R 5.	Before operation	Nov. 12	·114	·098	·123	·162	·144	·131	...	·117	...	·048	...
	After „	„ 22	·075	·048	·032	...	...	·037	·032*	...	...	...	·043
R 11.	Before „	„ 25	·076	...	·106	·102	·102	·097	...	·065	...	·030	...
	After „	Dec. 10	·099	·045	·060	·069	·056	·047	·049	·076	...	...	·054
R 22.	Before „	Jan. 8	·094	·094	·131	·146	·115	...	·122	...	...	·052	...
	„ „	„ 19	·082	·095	·153	·085	·071	·055	·051	...	...	·071	...
	After „	„ 28	·089	·084	·083	...	·078	·075	·067	...	...	...	·022
R 10.	Before „	Nov. 24	·113	·101	·143	·195	·174	·145	·117	...	...	·082	...
	After „	Dec. 7	·108	·095	·095	·095	·108	·075	·068	·072	·066	...	·042
	„ „	„ 10	·101	·083	·081	·089	·099	·069	·087	·076	·087	...	·032
	„ „	„ 13†	·087	·085	·079	·092	·085	·085	·087	·083	·078	...	·009
R 6.	After „	Nov. 17	·108	·110	·105	...	·110	·084	·086	·080	...	...	·028
	„ „	„ 19	·123	·110	·116	·109	·103	·086	·098	·105	...	...	·037
R 3.	„ „	„ 15	·131	·131	·139	·143	·132	·124	·074	·059*	...	...	·072
R 47.	„ „	April 11†	·110	·103	·102	·083	·082	·064	·049	·053	·033*	...	·077

* = Death. † = Experiment carried out under amytal anæsthesia.

TABLE II.—*Effect of Thyroidectomy and Thyroid Feeding on the Hyperglycæmic Reaction to Bacteria.*

		Date of injection.	Per cent. glucose in blood.		Maximum change in blood-sugar
			Before injection.	Maximum.	
R 1.	Before operation	Nov. 12	·114	·162	·048
	After „	„ 22	·075	·075	0
R 2.	Before „	„ 25	·076	·106	·030
	After „	Dec. 10	·099	·099	0
R 3.	Before „	Jan. 8	·094	·131	·052
	„ „	„ 19	·082	·153	·071
	After „	„ 28	·089	·089	0
R 4.	Before „	Nov. 24	·113	·195	·082
	After „	Dec. 7	·108	·108	0
	„ „	„ 10	·101	·101	0
	„ „	„ 13†	·087	·092	·009
R 5.	After „	Nov. 17	·108	·110	·028
	„ „	„ 19	·123	·123	0
R 6.	„ „	„ 15	·131	·131	0
R 7.	„ „	April 11†	·110	·110	0

† = Experiment carried out under amytal anæsthesia.

TABLE II.—*Effect of Thyroidectomy and Thyroid Feeding on the Hyperglycæmic Reaction to Bacteria.*

		Days after thyroidectomy.	Total gm. thyroid fed.	Date of injection.	Per cent. glucose in blood.		Maximum change in blood-sugar from preliminary level.
					Before injection.	Maximum change after injection.	
R 21.	Before thyroidectomy	...	...	Jan. 7	·084	·130	+ ·046
	After „	8	...	„ 20	·075	·090	+ ·015
	„ „	14	...	„ 26	·075	·068*	− ·007
R 31.	Before „	...	...	Feb. 2	·100	·198	+ ·098
	After „	5	...	„ 14	·084	·160	+ ·076
	„ „	12	...	„ 21	·115	·148	+ ·033
	„ „	20	...	„ 29	·103	·122	+ ·019
R 17.	Before „	...	...	Jan. 4	·105	·189	+ ·084
	After „	7	...	„ 13	·101	·161	+ ·050
R 22.	Before „	...	...	„ 8	·094	·146	+ ·052
	After „	7	...	„ 19	·082	·153	+ ·071
R 43.	„ „	11	...	Mar. 23	·080	·124	+ ·044
	„ thyroid feeding	19	4·75	April 2	·093	·091†	− ·002
R 23.	Before „ „	...	...	Jan. 10	·102	·126	+ ·024
	After „ „	...	0·42	„ 19	·090	·122	+ ·032
R 32.	Before „ „	...	...	Feb. 3	·110	·188	+ ·078
	After „ „	...	7·0	„ 11	·107	·149	+ ·042
R 44.	„ thyroidectomy	14	...	Mar. 28	·119	·100	− ·019
	„ thyroid feeding	22	1·75	April 5	·104	·163	+ ·059
	18 days after stopping thyroid feeding	39	...	„ 22	·106	·145	+ ·039
	33 days after stopping thyroid feeding	54	...	May 7	·105	·090	− ·015
	10 days after resuming thyroid feeding	...	1·50	„ 17	·110	·129	+ ·019
R 7.	Control. 1st injection	...	...	Nov. 9	·095	·178	+ ·083
	„ 2nd „	...	...	„ 11	·110	·182	+ ·072
R 38.	„ 1st „	...	...	Mar. 4	·120	·159	+ ·039
	„ 3rd „	...	...	„ 14	·082	·101	+ ·019

* This failure to respond was not due to lack of liver glycogen, as adrenaline was injected at the end of this experiment and caused the normal hyperglycæmic response.

† Excessive thyroid feeding. Liver was glycogen-free.

(c) *The Effect of Insulin.*

In one rabbit one unit of insulin given simultaneously with the bacterial injection neutralized the effect previously obtained in the same animal with bacteria alone.

(d) *Comparison with Adrenaline.*

In one rabbit the curve following bacterial injection was found to be roughly equivalent to the curve resulting from 0·4 mgm. adrenaline injected subcutaneously into the same animal.

(e) *Effect of Denervating the Liver.*

Denervation of the liver as an aseptic operation done 4 days previously in one animal did not prevent the usual hyperglycæmic reaction to bacteria, the blood-sugar rising in 1½ hours from ·108 to ·203%.

(f) *The Effect of Ergotamine Tartrate.*

Complete inhibition of the usual hyperglycæmic reaction resulted when 5 mgm. ergotamine tartrate was given simultaneously with the bacterial injection.

(g) *The Effect of Cutting the Vagus in Suprarenalectomized Rabbits.*

Britton (1925) showed that electric stimulation of the peripheral end of the right vagus nerve in cats, in which the influence of the suprarenals was removed, caused a lowering of blood-sugar which he attributed to stimulation of the islets. Clark (1925, 1926) and others have given evidence that the right vagus is concerned in the regulation of blood-sugar.

Since it was evident from our experiments that bacterial injections caused stimulation of the suprarenals by way of the sympathetic, it was thought that there might be an associated central stimulation of the islets by way of the vagus, which might account for the striking fall in blood-sugar after bacterial injections in animals which have been deprived of their suprarenals. However, when this theory was subjected to experimental trial, by cutting the right vagus in the neck (under amytal) before the bacterial suspension was injected in a suprarenalectomized rabbit, there was found to occur the usual fall in blood-sugar to a hypoglycæmic level.

To account for this fall we may conceive of several other possibilities:

(1) Direct, not central, stimulation of the islet cells.

(2) The fall may be due to increased utilization of glucose in the tissues, perhaps because the bacteria have stimulated the thyroid, which in turn has had its effect in accelerating tissue metabolism, and compensatory glycogenolysis to maintain the blood-sugar level is impossible in the absence of the suprarenals. The promptness of the response is against this view.

(3) The bacterial suspension may contain a thermostable substance with insulin-like action such as the thermostable substances described by Collip (1923) as glucokinin, present in yeasts and certain vegetable extracts. However, these act in the intact animal, whereas the bacterial suspension causes hypoglycæmia only in the absence of the suprarenals.

DISCUSSION.

The substance responsible for the hyperglycæmic effect of killed bacterial suspensions is thermostable, resisting exposure to 15 lb. pressure in the autoclave for 15 minutes.

The latent period of about 30 minutes before the reaction occurs suggests that the effective substance may be liberated from the tissues after introduction of the bacteria, or that the substance is contained within the bacterial body and must be liberated by lysis of the organisms. Menten has shown that, in the case of *B. paratyphosus* B and *B. enteritidis*, the substance causing hyperglycæmia occurs in protein-free filtrates of the organisms. Geiger does not state what his "colitoxin" is, but probably it is a filtrate of a broth culture. Presumably the filtrates of *B. proteus* and *B. coli* would be effective, in which case it is not likely that the response has any relation to the lysis of the organisms in the animal body. Furthermore, lysis of the organisms *in vitro* by autoclaving resulted in a curve exactly like that resulting from whole bacteria, with the same latent period. The delay then may be due to the liberation in the tissues of some substance, perhaps of a histamine-like nature, since histamine is known to produce a hyperglycæmia (Menten and Krugh, 1926; Chambers and Thompson, 1925; La Barre, 1926). The bacterial suspension itself may contain histamine, as Kendall and Schmidt (1926) found a histamine-like substance in cultures of the gas bacillus.

There is usually an increase in the rate and depth of respiration after injecting the bacterial suspension. This respiratory disturbance may play a *rôle*, but not an important one, as there is no parallelism between the amount of respiratory disturbance and the height of the blood-sugar. In some rabbits showing very little change in respiration there is a marked hyperglycæmia, while often those showing the most marked respiratory change are those rabbits showing very slight hyperglycæmia. In fact, the appearance is that of over-ventilation rather than asphyxia, and would, therefore, tend to lower rather than raise the blood-sugar concentration.

This bacterial hyperglycæmia does not occur in cats, and it is known that cats do not show definite anaphylactic phenomena. Perhaps bacterial hyperglycæmia is in some way associated with sensitization, especially since anaphylactic shock definitely causes hyperglycæmia (Zunz and La Barre, and others). Or the difference in reaction to bacteria in the rabbit and in the cat may be due to the rabbit's blood-sugar regulation being a more delicately balanced mechanism subject to the demands and emergencies of a different mode of life.

The effect of removing the suprarenals demonstrates that bacterial hyperglycæmia is the result of glycogenolysis, dependent upon adrenalin discharge through central stimulation by way of the splanchnics. That the hyperglycæmia is not a result of failure of utilization of glucose due to depressed activity of the islets is shown by the fact that after suprarenalectomy the bacterial injections induce a fall of blood-sugar even to a convulsive level. The islets, therefore, are probably functioning actively.

Cannon (1924), Houssay (1924) and others have shown that normally the injection of insulin results in a compensatory stimulation of the suprarenals. Perhaps this effect is accentuated in infection, since bacterial injections also

stimulate the suprarenals. The reduced effectiveness of insulin in diabetic patients suffering from infections would then be due to an excessive activity of the suprarenals. This seems a more plausible explanation than that the pancreas is affected by the infection, although the diabetic pancreas may be more susceptible to the influence of infection than the normal pancreas, and it would not be justifiable to apply too literally the results of such experiments on normal rabbits to the problem of human diabetes.

SUMMARY.

1. The hyperglycæmic response of rabbits to killed *B. coli* and *B. proteus* is due to central stimulation of the suprarenals by way of the splanchnic nerves, with consequent glycogenolysis.

2. Ergotamine tartrate prevents this hyperglycæmia, insulin neutralizes it.

3. There is suggestive evidence that thyroidectomy decreases the hyperglycæmic response to bacteria, and thyroid feeding increases it.

4. In rabbits with one suprarenal removed and the other denervated, the bacterial injection causes a lowering of blood-sugar, which may fall to a convulsive level, resulting in death. This hypoglycæmia is not likely to be due to central stimulation of the islets through the vagus, because the hypoglycæmia occurs after vagal section.

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OBSERVATIONS ON THE VIRULENCE OF HÆMOLYTIC STREPTOCOCCI.*

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In an earlier paper (1927) I described a method of measuring the power of hæmolytic streptococci to multiply in blood. The principle of this method is to incubate normal human defibrinated blood, infected with hæmolytic streptococci, on a mixing machine so that the leucocytes and cocci are mixed together during incubation. Equal volumes of infected blood are plated out in agar before incubation and after 3 hours' incubation on the mixing machine, and the number of colonies in the two plates are counted and compared. When old laboratory cultures are tested by this technique large numbers of streptococci are killed by the blood, but if freshly isolated strains are used the cocci multiply freely in blood. Table I shows the action of normal human blood on a freshly isolated strain, and on a strain which had been kept on artificial media for several months. Both these strains were isolated by blood culture from cases of puerperal septicæmia.

TABLE I.—*Compares the Power of Multiplication in Blood of an Old Laboratory Culture and a Freshly Isolated Culture.*

	Old laboratory culture.		Freshly isolated culture.	
Dilution of cultures.	Approximate number of cocci planted in 50 c.mm. of blood before incubation.	Number of colonies which grew out from 50 c.mm. of infected blood after 3 hours' incubation on mixing machine.	Approximate number of cocci planted in 50 c.mm. of blood before incubation.	Number of colonies which grew out from 50 c.mm. of infected blood after 3 hours' incubation on mixing machine.
1 in 50	14,800	112	15,000	+++
1 in 500	1,480	21	1,500	2,650
1 in 5,000	148	2	150	234

The numbers of streptococci planted in the blood from the two cultures were approximately equal, but while the organisms derived from the old culture were considerably diminished in number after 3 hours' incubation on the mixing machine, those derived from the freshly isolated culture had, under the same conditions, multiplied in the blood. This is a typical example of the difference between freshly isolated cultures and old laboratory cultures.

If a freshly isolated strain is put into Robertson's meat medium and after preliminary overnight incubation is kept at room temperature, broth cultures taken from the meat retain their power of multiplication in blood. But if the

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freshly isolated strain is subcultured in broth or on agar the cocci soon lose their power of multiplication in blood. As the most probable explanation of this change is loss of virulence, I have tested subcultures from freshly isolated strains with my own blood from day to day to see whether influences which are known to affect virulence will also affect the power of multiplication in blood. Freshly isolated strains have been subcultured in broth, on agar and in serum, and the cocci from these various cultures have been tested at frequent intervals for their power of multiplication in blood. Charts have been prepared showing the rise or fall in the power of the cocci to multiply in blood, and the relation of these changes to the medium in which the cocci were cultivated. Comparison has also been made between the power of streptococci to multiply in human blood and their virulence to mice. Before describing the results of these experiments it is necessary to refer to some technical points which must be observed if consistent results are to be obtained.

TECHNIQUE.

It is important to use cultures of the same age in each test, as young cultures are much more resistant to the bactericidal action of blood than older cultures. The following experiment shows that even a few hours' additional incubation of the culture prepared for a test will entirely alter the result of the experiment. A broth culture of a recently isolated strain of streptococcus was prepared, and after 16 hours' incubation the culture was divided into two parts. One part was kept on the bench at room temperature, and the other part was returned to the incubator. Five hours later the two parts of the culture were tested for their power of multiplication in blood (Table II).

TABLE II.—*Compares the Power of Multiplication in Blood of Two Samples of Cocci from the same Culture after Different Periods of Incubation.*

	16-hour broth culture.		21-hour broth culture.	
Dilution of cultures.	Approximate number of cocci planted in 50 c.mm. of blood before incubation.	Number of colonies which grew out from 50 c.mm. of infected blood after three hours' incubation on mixing machine.	Approximate number of cocci planted in 50 c.mm. of blood before incubation.	Number of colonies which grew out from 50 c.mm. of infected blood after 3 hours' incubation on mixing machine.
1 in 500	630	1248	810	281
1 in 5000	63	143	81	27

The culture which had been incubated for 16 hours and then kept on the bench at room temperature for 5 hours multiplied in the blood, but the culture which remained for the full 21 hours in the incubator had lost its power of multiplication in blood. This loss is not a permanent change, as fresh 16-hour broth cultures taken from the two cultures were approximately equal in their power of multiplication in blood. This is in agreement with the well-known observation that young cultures are more virulent than older cultures.

Another important point in observing changes in the power of streptococci to multiply in blood is that the blood should be taken each day from the same individual. This precaution is necessary, because variations in the bactericidal

action of one person's blood from day to day are small in comparison with the possible difference between samples of blood from two apparently normal persons. It will be seen from the accompanying curves that if these two precautions are observed fairly regular results can be obtained, but if different people's blood had been used from day to day to test cultures of various ages the results would have been chaotic.

All the cultures used in these experiments are puerperal strains which Dr. Colebrook isolated by blood-culture from cases of septicæmia, and 16-hour broth-cultures in Douglas's trypsin digest broth were used in each test. The characteristics of the strains will be described separately, with curves representing changes in the power of cocci from successive subcultures of the same strain to multiply in blood.

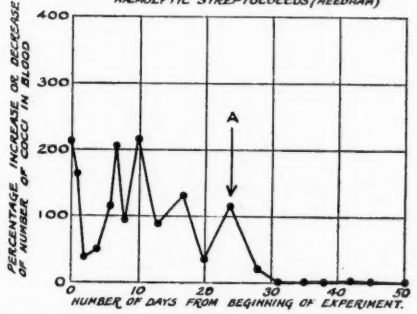
STRAIN I. (NEEDHAM.)

Chart 1 gives the power of multiplication in blood of successive subcultures of strain 1 in broth and on agar.

The ordinates are arrived at by calculating the percentage increase or decrease in the number of cocci in the blood after 3 hours' incubation on the mixing machine. Points on the 100-line represent no change in the streptococcal population of the blood; points below 100 represent decrease in population, and points above 100 represent increase in population. In other words, the curve records the numerical increase or decrease in blood of cocci from successive subcultures of the same strain.

CHART 1.

POWER OF MULTIPLICATION IN BLOOD OF SUCCESSIVE SUBCULTURES OF STRAIN 1. HÆMOLYTIC STREPTOCOCCUS (NEEDHAM)



The first test was done on a 16-hour broth culture of the freshly isolated strain which had been kept in Robertson's meat medium. The second subculture was tested the following day, and successive subcultures were tested at first daily and later at longer intervals. At the beginning of the experiment, when successive subcultures in broth were tested, the results were variable. At the point marked A the 24th subculture in broth was transferred to agar, and 16-hour broth cultures were taken from the agar at intervals and tested. Cultivation on agar caused a rapid fall in the power of the cocci to multiply in blood.

STRAIN II. (LONDON.)

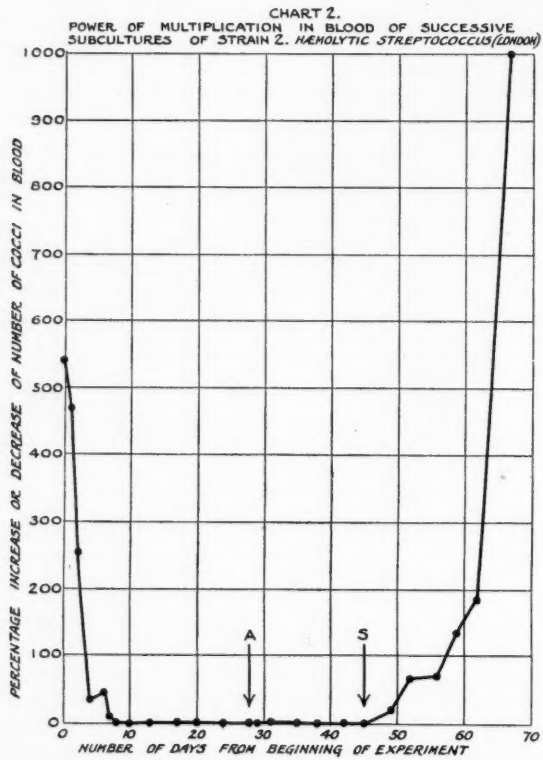
Chart 2 shows the same phenomenon with another strain of streptococcus, but in this case an increase in the power of multiplication in blood was obtained by cultivation in serum.

Cultivation in broth caused a very rapid fall in the power of the cocci to multiply in blood and the 7th subculture was down to the base-line. At A the culture was transferred to agar, and at S it was transferred to undiluted normal human serum. 16-hour broth cultures were taken from the agar and serum at intervals and tested. Cultivation in serum caused such an increase in the power of the cocci to multiply in blood, that after 12 subcultures in

serum they were able to multiply in blood more rapidly than at the beginning of the experiment.

The next experiment shows that cultures of this strain which were able to multiply rapidly in human blood were virulent to mice, whereas cultures which were killed by human blood were relatively avirulent to mice. The word "virulence" is used in this paper to express the power of a culture to kill

CHART 2.



an animal irrespective of the mechanism employed by the cocci. Virulence has been measured by inoculating falling dilutions of 16-hour broth cultures into mice. Experiments of this kind are not altogether satisfactory because of the great variation in the natural immunity of mice to infection with hæmolytic streptococci, but the method is sufficiently accurate to distinguish between highly virulent and attenuated cultures. Broth cultures from serum and agar were first tested for their power of multiplication in human blood and were then inoculated into mice. Table III compares the powers of multiplication in blood of 16-hour broth cultures taken from serum and agar. The former culture had been in serum for 110 days with 28 subcultures, the latter had been on agar for 68 days with 41 subcultures.

TABLE III.—*Compares the Power of Multiplication in Human Blood of Broth Cultures from Serum and Agar.*

Dilution of cultures.	16-hour broth culture of Strain 2 from serum.		16-hour broth culture of Strain 2 from agar.	
	Approximate number of cocci planted in 50 c.mm. of blood before incubation.	Number of colonies which grew out from 50 c.mm. of infected blood after 3 hours' incubation on mixing machine.	Approximate number of cocci planted in 50 c.mm. of blood before incubation.	Number of colonies which grew out from 50 c.mm. of infected blood after 3 hours' incubation on mixing machine.
1 in 50	28,000	++++	27,400	270
1 in 500	2,800	+++	2,740	38
1 in 5000	280	++	274	2
1 in 50,000	28	565	...	...
	Increase in streptococcal population = 2017·9%.		Average surviving streptococcal population = 1·0%.	

The culture from serum multiplied very rapidly during 3 hours' incubation in blood. On the other hand, the culture from agar was killed off under the same conditions. The percentage figures corresponding to those used in plotting the curves were 2017·9 and 1·0.

The two cultures were inoculated into mice with the results shown in the following table:

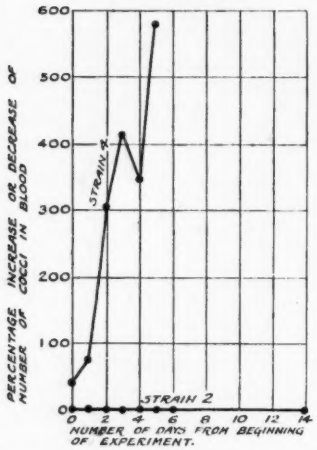
TABLE IV.—*Compares the Lethal Effect on Mice of Broth Cultures from Serum and Agar.**

	Dilution of cultures.								
	1 in 1.	1 in 10.	1 in 100.	1 in 1000.	1 in 10,000.	1 in 100,000.	1 in 1,000,000.	1 in 10,000,000.	1 in 100,000,000.
Sixteen-hour broth culture of Strain 2 from serum.	1	1	1	2	3	8	2	3	S
	1	1	2	3	3	S	S	S	S
Sixteen-hour broth culture of Strain 2 from agar.	1	S	S						
	1	S	S						
	1	S	S						

* Each square in the table represents a mouse. Each mouse received 0·25 c.c. of the cultures intraperitoneally in the dilutions indicated. The numbers in the squares give the days on which the mice died. S indicates that the mouse was alive and well 14 days after inoculation.

CHART 3

EFFECT OF SERUM PASSAGE ON TWO STRAINS OF HÆMOLYTIC STREPTOCOCCI AFTER CULTIVATION ON AGAR.



All the mice receiving undiluted culture from agar died, but all those receiving diluted culture survived. In contrast to this both the mice receiving culture from serum in a dilution of 1 in 10,000 died. The smallest dose of the culture from agar which killed a mouse was about 500,000,000 cocci. The smallest dose of the culture from serum which killed a mouse was about 50 cocci.

Experiments with this strain show that cultures which can multiply freely in human blood are virulent to mice, and this observation applies to all the strains of hæmolytic streptococci examined.

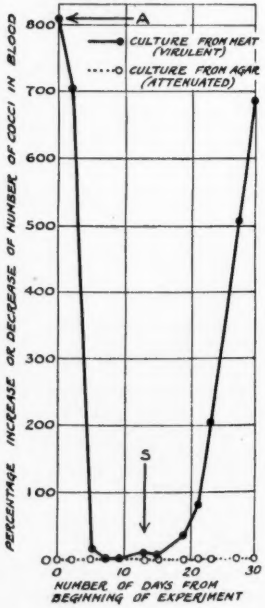
Conditions under which the virulence of Strain 2 can be increased by cultivation in serum.—The last experiment shows that the virulence of streptococci can be increased by cultivation in undiluted normal human serum. This response to cultivation in serum does not occur if the cocci have been cultivated on agar for a long time. Similarly after prolonged cultivation on agar transference to serum does not cause any increase in the power of the cocci to multiply in blood. The length of time required to bring about this indifference to serum passage varies greatly with different strains of streptococci. Chart 3 shows the effect of serum passage on Strain 2 and Strain 4 after they had each been subcultured daily on agar for 26 days. The 2 strains were subcultured into undiluted normal human serum, and 16-hour broth cultures were taken from the serum and tested daily.

This experiment shows that the power of multiplication in blood, tested *in vitro*, is not a true index of the virulence of streptococci, since it does not distinguish between cultures which have lost their power of response to serum passage and those which are still potentially virulent because their power of multiplication in blood can be restored by cultivation in serum. The following experiment shows that cocci which have lost their power of multiplication in blood may still be virulent to mice.

Two 16-hour broth cultures of Strain 2 were prepared—a virulent culture from meat and an attenuated culture from agar. These 2 cultures were injected into mice with the result shown in Table V, and their power of multiplication in blood

CHART 4

EFFECT OF AGAR AND SERUM PASSAGE ON VIRULENT AND ATTENUATED CULTURES OF STRAIN 2.



was tested on the same day. They were then subcultured on agar, and 16-hour broth cultures from the agar were tested at intervals for their power of multiplication in blood. Chart 4 gives the results of the tests.

The dotted line represents the attenuated culture and the continuous line represents the virulent culture. When the two cultures were approximately equal in their power of multiplication in blood, at the point marked S on the chart they were again injected into mice, and were then subcultured into undiluted normal human serum. The virulent culture which had lost its power of multiplication in blood responded to serum passage as shown by the curve, but the attenuated culture did not recover its power of multiplication in blood. Tables V and VI give the lethal effect of these cultures on mice at the points marked A and S on Chart 4.

TABLE V.—*Lethal Effect on Mice of Virulent and Attenuated Cultures of Strain 2.**

	Dilution of cultures.					
	1 in 1.	1 in 10.	1 in 100.	1 in 1000.	1 in 10,000.	1 in 100,000.
Virulent culture at point marked A on Chart 4 (multiplied rapidly in human blood).	1	1	1	2	2	2
	1	2	2	**S**	3	2
Attenuated culture at point marked A on Chart 4 (did not multiply in human blood).	1	**S**	**S**	**S**	**S**	**S**
	2	**S**	**S**	**S**	**S**	**S**

* For explanation of arrangement of table see Table IV.

Table V shows that the culture from meat with a high power of multiplication in blood killed 11 of the 12 inoculated mice within 3 days. The attenuated culture of the same strain which had lost both its power of multiplication in blood and its power of response to serum passage killed only 2 out of 12 mice.

Table VI shows the lethal effect of the same 2 cultures after 12 daily subcultures on agar when they were approximately equal in their power of multiplication in blood, the only difference between the cultures being that one had lost its power of response to serum passage, while the other retained this property. The culture responsive to serum passage killed 7 out of 12 mice. The attenuated culture again killed 2 mice. Although there was a definite decrease in the virulence of the culture from meat after 12 passages on agar, it was still much more virulent than the attenuated culture in spite of the fact that these two cultures were about equal in their power of multiplication in blood. Steps were therefore taken to see whether the culture which responded to serum passage *in vitro* had undergone a similar change in the bodies of the mice.

TABLE VI.—*Lethal Effect on Mice of the Same Virulent and Attenuated Cultures of Strain 2 after 12 Days on Agar.*

	Dilution of cultures.					
	1 in 1.	1 in 10.	1 in 100.	1 in 1000	1 in 10,000.	1 in 100,000.
Virulent culture at point marked S on Chart 4 (did not multiply in human blood).	1	2	6	3	**S**	8
	1	3	**S**	**S**	**S**	**S**
Attenuated culture at point marked S on Chart 4 (did not multiply in human blood).	1	**S**	**S**	**S**	**S**	**S**
	6	**S**	**S**	**S**	**S**	**S**

16-hour broth cultures were prepared from the heart's blood of all the dead mice shown in Table VI, and were tested for their power of multiplication in human blood. Table VII gives the result of this experiment, the power of multiplication in blood being expressed as the percentage increase or decrease in the streptococcal population of human blood after 3 hours' incubation on the mixing machine:

TABLE VII.—*Effect of Mouse Passage on Virulent and Attenuated Cultures of Strain 2.*

Description of cultures before inoculation into mice. (From Chart 4.)	Dilution of culture inoculated into mice. (From Table VI.)	Number of days between inoculation and death of mice. (From Table VI.)	Power of multiplication in human blood after passage through mice.
Attenuated culture at point marked S on Chart 4 (Power of multiplication in blood lost and power of response to serum passage lost)	Undiluted	1	1·5 (% of survivors in blood).
	„	6	1·1 (ditto).
Virulent culture at point marked S on Chart 4 (Power of multiplication in blood lost but power of response to serum passage retained)	Undiluted	1	91·6 (% of survivors in blood).
	„	1	19·6 (ditto).
	1 in 10	2	283·0 (% increase of streptococcal population in blood).
	1 in 10	3	263·5 (ditto).
	1 in 100	6	420·3 „
	1 in 1000	3	150·0 „
	1 in 100,000	8	144·5 „

The attenuated culture which had lost its power of response to serum passage *in vitro* did not regain its power of multiplication in blood after passage through mice. On the other hand, the virulent culture which had lost its power of multiplication in blood, but had retained its power of response to serum passage, underwent the same change in mice as in serum, and regained its power of multiplication in blood. In addition to the figures given in Table VII, cultures from 5 mice which had died after inoculation with attenuated cocci of Strain 2 were tested, but none of these cultures had regained their power of multiplication in blood.

Experiments with all the strains of streptococci examined show that cultures which are capable of rapid multiplication in human blood are virulent to mice, but cultures which are killed by human blood give variable results when inoculated into mice. The last experiment possibly explains why cultures which have lost their power of multiplication in blood *in vitro* differ in their virulence to mice. Such cultures may be divided into two classes: (1) Those which are virulent because they are capable of regaining their power of multiplication in blood after inoculation. (2) Those which are attenuated because they cannot regain their power of multiplication in blood after inoculation. These two classes can be recognized *in vitro* by testing their power of response to serum passage.

STRAIN III. (CRUDGINGTON.)

Similar results were obtained with Strain 3, the virulence of the cocci being increased by cultivation in normal human serum. After cultivation on agar, the cocci, having lost their virulence to mice and their power of multiplication in human blood, passed into a condition in which their virulence could not be restored by cultivation in serum.

STRAIN IV. (HENSON).

Chart 5 shows the effect of cultivating Strain 4 in broth, on agar and in serum.

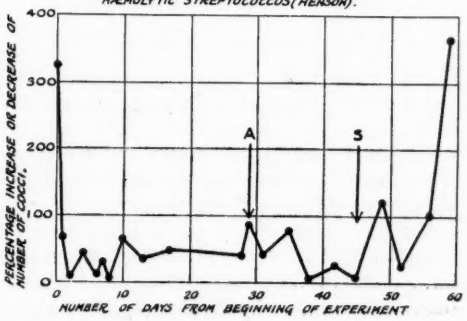
The outstanding characteristic of this strain is its tenacity in maintaining its power of response to serum passage after prolonged cultivation on agar, and this characteristic is possibly associated with its fluctuating power of multiplication in blood after growth on agar. The following comparison of the time required to attenuate 3 strains by cultivation on agar shows that Strain 4 is attenuated more slowly than the other 2 strains:

Strain 3 had lost its power of response to serum passage after 47 days on agar.

Strain 2 had lost its power of response to serum passage after 79 days on agar.

CHART 5.

POWER OF MULTIPLICATION IN BLOOD OF SUCCESSIVE SUBCULTURES OF STRAIN 4. HÆMOLYTIC STREPTOCOCCUS (HENSON).



Strain 4 had *not* lost its power of response to serum passage after 143 days on agar.

I found that the power of multiplication in blood could be rapidly reduced by growing this strain in normal human blood-cells which had been washed with salt solution to remove the serum. The cells were prepared by centrifuging defibrinated blood and removing the serum. After three washes with normal saline, the last wash of salt solution was pipetted off and the streptococci were inoculated into the packed cells at the bottom of the tube. When this streptococcus had been cultivated for nearly a year in undiluted serum and in washed cells there was a very marked difference between broth cultures taken from the serum and cells.

The culture from serum multiplied rapidly in human blood, the percentage figure corresponding to those used in plotting the curves was 2000, and this culture killed a mouse in a dilution of 1 in 100,000. On the other hand, the cocci from washed cells were destroyed by human blood (percentage figure 0·3), and they failed to kill a mouse in a dilution of 1 in 10.

The culture from cells had probably lost its power of response to serum passage, but unfortunately this point was not investigated until a considerable time after the above experiment. When the culture had been in washed cells for more than a year the cocci had lost their power of response to serum passage.

Strain 4 has the same characteristics as the other strains. The power of multiplication in blood is lost by cultivation on agar, and is restored by cultivation in serum. The power of response to serum passage is maintained on agar for an unusually long time, but as this property is lost after cultivation in washed blood-cells it can probably be abolished by longer cultivation on agar.

The examination of these 4 strains brings out several points of interest in the study of virulence: (1) All the cultures which were able to multiply freely in fresh human blood were virulent to mice. (2) All the cultures which had lost their power of multiplication in blood were exalted by cultivation in serum if they had not been kept too long on agar. (3) All the cultures which had been kept on agar long enough to abolish their power of response to serum passage were attenuated. (4) The time required to attenuate different strains by cultivation on agar was very variable.

GENERAL CHARACTERISTICS OF EXALTED AND ATTENUATED CULTURES.

Virulent and attenuated cultures derived from the same original blood-culture are morphologically indistinguishable with chains of the same length and cocci of the same size. The colonies on blood-agar plates are similar in appearance after 36 hours' incubation, but colonies of highly virulent cocci develop rather more slowly on agar than colonies of attenuated cocci, so that after 16 hours' incubation virulent colonies are smaller than attenuated colonies. Emulsions of virulent cocci agglutinate spontaneously in salt solution and in distilled water, but attenuated cultures give relatively stable emulsions in both salt solution and water. This is in accordance with the observation made by Eagles (1926) that recently isolated cultures of hæmolytic

streptococci make unsuitable emulsions for serum agglutinations, because they agglutinate spontaneously.

Virulent cultures are resistant to phagocytosis. Table VIII gives the result of a phagocytic experiment with virulent and attenuated cultures of the same strain. The 16-hour broth cultures used in this experiment were of equal density, and when plated out in agar gave similar counts.

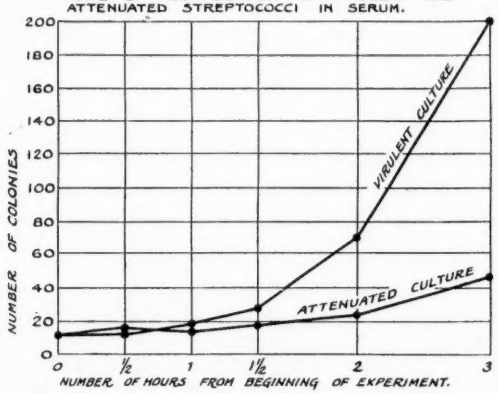
TABLE VIII.—*Phagocytosis of Virulent and Attenuated Cultures of the same Strain.*

	Leucocytes containing streptococci.	Leucocytes containing no streptococci.
Virulent culture . . .	15	185
Attenuated culture . .	102	98

Two parts of defibrinated blood were incubated for 15 minutes with one part of each of the cultures. The number of leucocytes containing streptococci were counted, 200 leucocytes being counted in each case.

CHART 6.

RATE OF MULTIPLICATION OF VIRULENT AND ATTENUATED STREPTOCOCCI IN SERUM.



Only 15 leucocytes contained virulent streptococci, but 102 contained attenuated cocci. This experiment was repeated with cultures which had been killed by heat at 60°C. for 1 hour and the same result was obtained. The resistance of virulent cocci to phagocytosis can be shown in another way. If a sufficiently virulent culture is selected, the cocci multiply with equal rapidity in defibrinated blood and in serum—an observation which shows that leucocytes have no power to check the rate of multiplication of highly virulent streptococci.

Virulent cultures are resistant to phagocytosis, but this is not the whole explanation of why they multiply in defibrinated blood. The two cultures also differ in their rate of multiplication in serum. Chart 6 shows the rate of multiplication of virulent and attenuated cultures of the same strain in fresh human serum.

A sample of serum was divided into two parts which were infected with equal volumes of diluted broth cultures of the virulent and attenuated cocci.

The two infected sera were incubated on the mixing machine, and equal volumes were plated out in agar before incubation and after ½, 1, 1½, 2 and 3 hours' incubation. The ordinates are the number of colonies counted in the plates and the abscissæ give the time. The virulent culture is resistant to phagocytosis and also has the power of rapid multiplication in serum.

It was stated above that colonies of attenuated cocci develop more rapidly on blood-agar plates than colonies of virulent cocci. On the other hand, we have just seen that virulent cocci multiply more rapidly in serum than attenuated cocci. These two observations may possibly explain why cultures become attenuated on agar and exalted in serum. Cultivation on agar may select the more rapidly growing, relatively saprophytic cocci, and cultivation in serum may select the more rapidly multiplying virulent cocci. In this connection it is interesting to note that my colleague, Dr. Fleming, has observed that if virulent and attenuated cultures of the same strain are plated out on blood-agar, poured at 80°C., there is often a distinct difference between the colonies. The plate spread with virulent culture contains a large proportion of colonies with central eminences, matt surfaces and clearly defined margins which, being subcultured in broth, grow in granular masses at the bottom of the tube. The plate spread with attenuated culture contains a large proportion of rounded, glossy colonies with well-defined edges which produce uniform turbidity in broth. These two types of colonies are not the same as the "rough" and "smooth" colonies described by Cowan (1922). In fact, the relationship between colony formation and virulence is reversed, as cultures with matt colonies which in some respects resemble her "R" attenuated cultures are the virulent type.

Although the matt colonies described above are a well-marked characteristic of virulent cultures, it is improbable that the exaltation of virulence which follows cultivation in serum is entirely due to selection of this type of coccus, because matt colonies sometimes retain their distinctive characteristics of colony formation and granular growth in broth after prolonged cultivation on agar but lose their power of multiplication in blood. In addition to the matt and glossy colonies already described, an intermediate type of colony with matt surface, but without the central eminence, is frequently seen. The exact significance of these different types of colonies in relation to virulence is at present uncertain, but it seems probable that the glossy colonies are composed of cocci which are completely attenuated and cannot be restored to a virulent state by cultivation in serum, while the matt colonies are composed of cocci which are either virulent or can be made virulent by cultivation in serum.

A NOTE ON STREPTOCOCCAL TOXIN AND ITS RELATION TO VIRULENCE.

Since the Dicks demonstrated the presence of a soluble toxin in filtrates from cultures of *Streptococcus scarlatinæ*, a number of observers have found that a similar toxin can be isolated from *Streptococcus pyogenes*. Eagles (1926), who examined about 30 strains of hæmolytic streptococci from cases of puerperal septicæmia, found that a number of these strains produced a toxin indistinguishable from scarlet fever toxin. It is therefore important to know what influence the toxin has on the power of the cocci to multiply in blood and to kill mice. Strains 2 and 4 described above were examined by Eagles for

their power of producing soluble toxin. Strain 2 was the most highly toxigenic of the 29 puerperal strains he examined, but he was unable to isolate toxin from strain 4. As the power of these two strains to multiply in blood and to kill mice appeared to be entirely independent of their toxin production, it was decided to test a strain which was capable of producing a more powerful toxin. Dr. O'Brien, of the Wellcome Research Laboratories, kindly supplied me with a culture of Dochez's *Streptococcus scarlatinæ* and with toxin prepared from the same strain. Intradermal injection of the toxin into my skin caused a strongly positive reaction, showing that my serum did not contain antitoxin. The power of Dochez streptococcus to multiply in my blood was then tested, and it was found to be very susceptible to the bactericidal action of leucocytes (percentage of survivors, 0·4).

An attempt was then made to raise the virulence of this strain by cultivation in serum, but, as might be expected from the length of time since the strain was isolated, it had lost its power of response to serum passage. 1 part of the undiluted toxin was then added to 39 parts of my blood and the power of Dochez streptococcus to multiply in this blood was again tested. Even after the addition of this large quantity of toxin the cocci were unable to multiply in the blood.

Two series of mice were inoculated with 0·25 c.c. of a 16-hour broth culture of Dochez streptococcus in dilutions ranging from undiluted culture to 1 in 100,000.

All the mice were alive and well 14 days after inoculation. These experiments, coupled with those already described with the toxigenic Strain 2 and the atoxic Strain 4, seem to indicate that a highly toxigenic streptococcus which has become attenuated by prolonged cultivation on artificial media is relatively harmless in spite of its unimpaired power of producing toxin.

SUMMARY AND CONCLUSIONS.

Hæmolytic streptococci freshly isolated from the blood-stream have the power of rapid multiplication in normal human blood and are virulent to mice.

Cultivation on agar causes a rapid fall in the power of the cocci to multiply in blood, but this lost characteristic can be restored by cultivation in normal human serum.

After more prolonged cultivation on agar the cocci pass into a state in which cultivation in serum does not restore the power of multiplication in blood.

The process of attenuation may therefore be divided into two stages: stage 1, in which the cocci, having lost their power of multiplication in blood, can have this property restored by cultivation in serum; stage 2, in which the power of multiplication in blood cannot be restored by cultivation in serum.

Experiments on mice show that highly virulent cultures which have been freshly isolated or have had their virulence enhanced by cultivation in serum will kill mice in a few days even when small numbers of cocci are injected. Subcultures in stage 1 of attenuation kill mice almost as readily as freshly isolated strains, and the cocci recovered from the mice have had their power of multiplication in human blood restored. Cultures in stage 2 of attenuation are relatively harmless to mice, and cocci recovered from the mice are still unable to multiply in blood.

The virulent and attenuated cultures described in this paper are not the same as "rough" and "smooth" cultures described by Cowan.

Virulence is not dependent on the production of soluble toxin.

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SIMULTANEOUSLY DETERMINED ARTERIAL AND VENOUS BLOOD-SUGARS AND RESPIRATORY QUOTIENT-TIME CURVES OF DIABETICS FOLLOWING INSULIN ADMINISTRATION.

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THE view generally held now is that glycogen formation is a necessary preliminary step to the oxidation of glucose in the body. Though this has not been definitely established, proof of it is accumulating. It is not the intention to review the literature which exists on this subject, but to present more evidence in favour of this idea in the nature of clinical studies. This consists of simultaneously determined arterial and venous blood-sugar and respiratory quotient-time curves of diabetic individuals following intravenous administration of insulin.

Data bearing on this problem have been reported previously from these laboratories (1925). These consisted of simultaneously determined venous blood-sugar and respiratory quotient-time curves of normal individuals, renal glycosurics and diabetics following glucose ingestion. These will be referred to briefly.

The responses of non-diabetic individuals to glucose ingestion were not uniform. In one group of cases increases of the respiratory quotients, indicating oxidation of carbohydrates, were observed shortly after ingestion of this sugar, whereas in another group, not only were no such increases noted, but actual decreases of the respiratory quotients occurred. An analysis of these cases showed that those individuals whose respiratory quotients did not increase had been on low carbohydrate diets prior to the tests. Since the blood-sugar data of each of these cases indicated that, at least, part of the glucose ingested was absorbed, and the urinary analysis showed it was not excreted, the conclusion was that the glucose ingested must have been stored.

In diabetics, it was shown that the respiratory quotient after glucose ingestion is not necessarily an index of the severity of the disease. In

moderately severe diabetes the respiratory quotients may be increased after glucose, whereas in mild diabetes, not only may there be no such increases, but, as in non-diabetics, actual decreases may be noted. Here also blood and urinary sugar data indicate that at least part of the glucose ingested was stored.

From the above observations it would appear that, when the glycogen stores of the body are depleted, glycogen formation, and not oxidation, is the first response to glucose ingestion. That glycogen formation is a necessary preliminary to glucose oxidation is suggested from the recent work of Lawrence (1926), which tends to prove that the action of insulin is not catabolic but anabolic; that it forms glycogen from glucose, but does not burn glucose directly; that insulin abolishes ketosis, not by burning carbohydrate directly, but by providing it in an *oxidizable* form.

The value of such methods as those employed in this investigation becomes obvious from the following considerations. In the observation that, even in a normal individual, glucose ingestion does not necessarily result in glucose oxidation, we have another demonstration of the fact that the different food materials (carbohydrate, fat and protein) ingested during the course of an experiment are not necessarily those oxidized, either in amounts or in proportions. This further emphasizes the importance of respiratory quotient studies in such experiments.

The importance of simultaneously determined arterial and venous blood-sugars may also readily be seen. It has been shown (Foster, 1923) that in normal individuals following glucose ingestion the concentration of glucose in the arterial blood reaches a higher level than in the venous specimen. This is the result of oxidation by or storage in the tissues. This knowledge was first applied clinically in diabetes by Lawrence (1924), and subsequently by one of the writers (Rabinowitch, 1927), who showed that the magnitude of the difference between the arterial and venous blood-sugars after glucose ingestion is a function of the severity of the disease. These observations indicated that in the absence of respiratory quotients the venous blood-sugar alone yielded little information as to the course of ingested glucose.

That a combined study of respiratory quotients and arterial and venous blood-sugars is the ideal method becomes obvious when we recognize the possible events after glucose ingestion. A proper interpretation of such events is only possible by means of such a study. Thus (*a*) a rise in the blood-sugar concentration after glucose ingestion indicates that at least part of the glucose has been absorbed from the alimentary canal; (*b*) the finding of an arterial blood-sugar higher than the venous indicates that the blood leaving the tissues has less sugar than that entering them—that is, part of the sugar ingested has been oxidized or stored; and (*c*) the respiratory quotient indicates which of the latter has taken place.

METHOD OF INVESTIGATION.

Respiratory quotients were first obtained in the fasting state. Arterial and venous blood specimens were then collected. Insulin, in 25-unit doses, was given intravenously, and respiratory quotients and arterial and venous blood-sugar estimations were made one hour, and, in a few instances, two hours

Arterial and Venous Blood-Sugar Percentages and Respiratory Quotients before and after Administration of Insulin.

No.	Fasting period.				One hour after insulin.				Two hours after insulin.			
	Blood-sugars.			R.Q.	Blood-sugars.			R.Q.	Blood-sugars.			R.Q.
	Arterial (A.).	Venous (V.).	A.—V.		Arterial (A.).	Venous (V.)	A.—V.		Arterial (A.).	Venous (V.).	A.—V.	
1	0·263	0·285	−0·022	·714	0·242	0·238	+0·004	·720	0·216	0·184	+0·032	·79
2	0·248	0·257	−0·009	·723	0·211	0·175	+0·036	·729	...	...	...	...
3	0·315	0·332	−0·017	·701	0·295	0·285	+0·010	·714	0·232	0·198	+0·034	·764
4	0·212	0·218	−0·006	·729	0·203	0·189	+0·014	·731	...	...	...	...
5	0·239	0·253	−0·014	·695	0·227	0·224	+0·003	·712	0·200	0·181	+0·019	·810
6	0·218	0·216	+0·002	·756	0·197	0·152	+0·045	·832	...	...	...	...
7	0·314	0·310	+0·004	·722	0·226	0·203	+0·023	·729	...	...	...	...
8	0·260	0·268	−0·008	·741	0·214	0·168	+0·046	·739	...	...	...	...
9	0·416	0·421	−0·005	·687	0·367	0·344	+0·023	·718	0·306	0·282	+0·024	·771
10	0·250	0·248	+0·002	·743	0·219	0·198	+0·021	·784	...	...	...	...

after. The general technique has been described previously (Rabinowitch, 1925 and 1927).

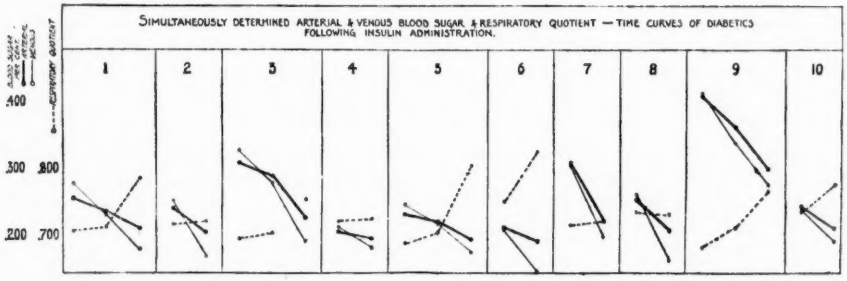
Very severe diabetics were selected purposely, in order, if possible, to obtain fasting bloods in which the sugar values of the venous bloods were higher than those of the arterial specimen.

Insulin was given intravenously in order to ensure a rapid fall of the blood-sugar, since the rate of fall of the blood-sugar depends upon the method of administration, being most rapid by the intravenous, slower with subcutaneous, and slowest with intradermal route.

In the interpretation of the data the following assumptions have been made, which, on the basis of previous work (Rabinowitch, 1927), have been proven to be correct:

(*a*) A fasting venous blood-sugar higher than that of the arterial blood indicates severe diabetes.

(*b*) If the arterial blood-sugar is higher than that of the venous blood, it indicates utilization of carbohydrates by the tissues. (By utilization is meant the phenomenon of oxidation or storage or both of these.) The combined data obtained in ten cases are shown in the accompanying table. These are graphically recorded in the chart.



DISCUSSION OF RESULTS.

With three exceptions (Cases 6, 7 and 10), all patients were proven to be severe diabetics—that is, the venous blood-sugars were higher than those of the arterial specimens in the fasting state. The simultaneously determined arterial and venous blood-sugar indicated that the severity of the disease in each of these cases was such that the glucose derived from the protein metabolism was not readily utilized nor stored as glycogen, and accumulated on the venous side of the circulation. In the three exceptions, the arterial and the venous blood-sugars were practically the same in the fasting state. Differences of 1 to 4 mgm. are discounted, as such could readily be the result of technical difficulties.

Insulin administration was effective in lowering the blood-sugars in all of the cases at the end of the first hour. In two cases only (Nos. 6 and 10) could it be definitely stated, from respiratory quotient observations, that the oxidation of glucose was partly responsible for this phenomenon. In other words, the

first effect of the insulin was to enable storage or conversion of glucose into glycogen. This view is supported by the fact that in practically every case at the end of one hour the arterial blood-sugars were higher than those of the venous specimen. In the two possible exceptions (Nos. 1 and 5), in which the arterial and venous blood-sugars were identical (within the limits of experimental error), one hour after insulin administration the phenomenon of storage appears also to have been demonstrated, since the venous blood-sugars were higher than those of the arterial specimen before the insulin. The two cases (Nos. 6 and 10) which showed definite evidence of oxidation at the end of the first hour are probably not as severe as the others, since the arterial and venous blood-sugars were identical in the fasting state. In all of the four cases in which observations were also made, two hours after insulin administration, definite evidence of oxidation of glucose was obtained. Since no such evidence was found at the end of the first hour, we again note storage taking place prior to oxidation.

SUMMARY.

In a series of ten patients, eight of whom were severe diabetics, who were unable to store glucose, and had low respiratory quotients in the fasting state, the first effect noted after the administration of insulin was storage and not oxidation of glucose. In the two cases in which oxidation was the first phenomenon noted, the arterial and venous blood-sugars were identical, and they therefore were probably not as severe diabetics as the others, and therefore subjects unfit for this investigation. A reasonable assumption is that these two subjects had sufficient glycogen stored, and were therefore able to oxidize it. The evidence obtained here, therefore, supports the view that glycogen formation is a necessary preliminary to oxidation of carbohydrates.

Clinically there is much which supports this view. It is well known that diabetics, in coma, react differently to insulin, in that the rates of recovery from this condition differ. Some do not recover at all. A characteristic finding in the latter group of cases is the persistence of a venous blood-sugar higher than that of the arterial specimen. In other words, glycogenesis from glucose derived from the protein metabolism does not take place. Since glycogenesis does not take place, oxidation does not take place. Since oxidation does not take place, the patients do not recover. Many variables are recognized which tend to prevent glycogenesis, and one, or a combination of these, is almost invariably found in such cases. These include fever, suppuration, stimulation of the Claude Bernard centre resulting from hæmorrhage, meningitis, etc.

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THE RELATION OF HYDROGEN ION CONCENTRATION TO THE ACTION OF THE LOCAL ANÆSTHETICS.

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GROS (1912), Regnier (1924–1925), Copeland (1925) and others have shown that alkaline solutions of local anæsthetics are active in higher dilutions than acid solutions. Many other salts of physiologically active bases are also potentiated by alkali in the same way, *e. g.* quinine (Brown and Henry, 1923).

Gros suggests that the free base is the only active constituent, and that alkalinization produces increased anæsthetic action by virtue of the increase in the amount of base set free. He suggests that in the form of free base, all anæsthetics are much of the same activity, and that a correlation exists between the local anæsthetic action and the solubility of the base in lipoid solvents.

Regnier (1925[2]), on the other hand, on the ground that the addition of alkali to a saturated solution of cocaine base in water increases the anæsthetic action, considers that all the forms, ion, base and salt in which cocaine occurs in aqueous solution are active, and that the action of alkali is directly on the tissues concerned.

We have investigated the action of a series of local anæsthetics on the rabbit's cornea under slightly different conditions, and have determined the dissociation constants with a view to comparing the relative activities of the free bases at the same hydrogen-ion concentration.

The experiments have been carried out with solutions of the hydrochlorides of the base dissolved in Clark and Lub's buffer mixtures. In the stronger solutions necessary at the more acid reactions, the reaction of the buffer was shifted towards the acid side. The actual reaction was estimated colorimetrically. The smallest concentration of anæsthetic which will produce anæsthesia in ten minutes was determined at each pH, the necessary dilutions being carried out with buffer solutions. The time limit of ten minutes was adopted because it was found that further dilution beyond the concentration necessary to produce anæsthesia in that time did not cause anæsthesia at all (see Fig. 1). The dilutions were tested in a series, each dilution being half the concentration of the previous one, and the end-point taken as that which produced a patchy anæsthesia in ten minutes, or alternatively, that concentration intermediate between that which produced complete anæsthesia in rather less than ten minutes and that which produced no anæsthesia at all. It is of great importance, for the reasons stated below, to use heavily buffered solutions, and to instil the solution continuously during the specified time, testing the response of the corneal reflex to light touch during instillation.

The effect of hydrogen-ion concentration has been examined for cocaine,

novocaine, β-eucaine, stovaine, conessine, benzylbenzoyl-ecgonine and phenylethylbenzoylecgonine. The latter two compounds were synthesized for the first time by Gray (1925). The results are plotted in Fig. 2, where the ordinates give the logarithms of the minimum molar concentrations of anæsthetic necessary to produce anæsthesia, and the abscissæ the pH. The dots give the actually observed points of the experiments with cocaine, which were typical of the series. Fig. 2 shows that over a large range of concentration the relation between the minimum concentration required to obtain anæsthesia is linear, but in the neighbourhood of pH 7 to 8 diminution of the pH commences to have less effect, so that the curves become convex towards the abscissæ. The straight parts of these curves are parallel, with the exception of that for conessine, the straight part of which has approximately twice the slope of the other.

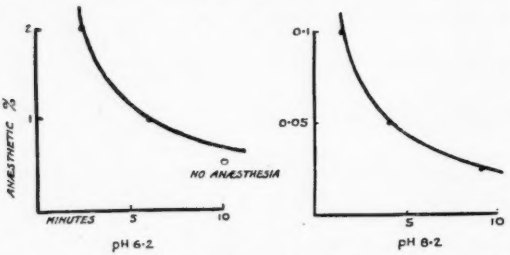

FIG. 1.—Curves showing times of onset of anæsthesia with novocain at different pH's.

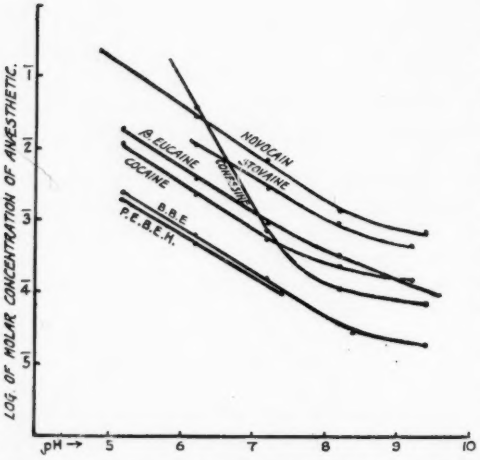

FIG. 2.—Curves showing the relation between anæsthesia and pH.

The reason for choosing a logarithmic scale for the concentrations is that the concentrations distinguishable are members of a geometrical series. It is just as easy to distinguish 0·005% from 0·01% cocaine at pH 8·2, as it is to distinguish 0·5% from 1% at pH 5·2.

To estimate how far these results agree with Gros's hypothesis that the active constituent of a solution of cocaine is the base, necessitates the determination of the amount of undissociated base present in the solution at each hydrogen-ion concentration. We have worked out electrometrically with the glass electrode (Kerridge, 1926) the neutralization curves for the substances used in this investigation. The results are given in Fig. 3. The solutions are M/300 in concentration, stronger solutions of some of the substances (benzylbenzoyl-ecgonine-hydrochloride, for example) giving precipitates of base with very small additions of soda. 10 c.c. quantities were titrated with 0·56N soda (free from carbon dioxide), the soda being added from a micrometer syringe (Trevan, 1925), volume changes during the titration being, therefore, reduced

to negligible amounts, since 10 c.c. of M/300 required only about 0·03 c.c. of 0·56 N soda for half neutralization. This amount can be measured with the micrometer syringe to 1 part in 300, which is more than sufficient for the purpose—a 1% error in the amount of soda added for half neutralization giving an error in the estimate of pK of 0·01 of a pH unit. Each point on the curve was estimated on a separate sample of the stock solution, the requisite amount of soda being added to every sample immediately before the determination of its pH. The precipitation of base prevents the working out of the whole titration curve with several of these substances, but sufficient of the titration curve can be obtained for the half-neutralization point to be determined, except for phenylethylbenzoylecgonine hydrochloride. The pH at this point

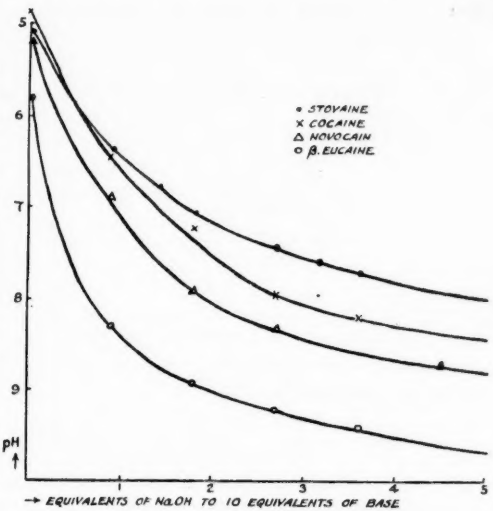

FIG 3.—Titration curves of anæsthetics.

TABLE I.—*Effect of Reaction on the Anæsthetic Action of Cocaine on the Cornea of the Rabbit.*

pH.	Concentration.	Time for anæsthesia.	End-point.
5·2	1·0%	4′+	
	0·5%	9′+	
	0·25%	10′—	0·375%
6·2	0·15%	4′+	
	0·075%	10′±	0·075%
7·2	0·2%	1′+	
	0·1%	1′+	
	0·05%	1′+	
	0·05%	1′+	
	0·025%	7½′+	
	0·025%	7½′+	
	0·0125%	10′—	0·019%
8·2	0·01%	7′+	
	0·005%	10′—	0·0075%
9·4	0·01%	4½′+	
	0·005%	108′—	0·0075%

when subtracted from 14·14 gives the pK (negative log of dissociation constant) with sufficient accuracy for our purposes. Fig. 3 gives the titration curves, and Table II the estimated pK for each. There is sufficient agreement between the estimated pK for novocaine and cocaine obtained by us and those obtained by Kolthoff (1925).

TABLE II.—*Dissociation Constants (pKs) for Anæsthetics.*

M/300	Stovaine	6·14
„	Cocaine	5·69 (Kolthoff gives 5·59).
„	B.B.E.	5·69
„	Novocain	5·29 („ „ 5·15).
„	β-Eucaine.	4·49
„	Conessine	pK_1 *ca* 4·6

If only undissociated base is active, the amount of base present in the limiting active concentrations at different pH's will be the same. The concentrations of anæsthetic which contain equal quantities of base can be calculated from the equations:—

$$[B^+] \times [OH^-] = K \times [BOH] \quad . \quad . \quad . \quad 1$$
$$[B^+] + [BA^-] + [BOH] = C \quad . \quad . \quad . \quad 2$$

where C is the limiting molecular concentration to produce anæsthesia at any hydroxyl ion concentration [OH] (the square brackets represent molecular concentration), $[B^+]$ is the molecular concentration of basic ion, [BA] that of salt. As a first approximation [BA] may be ignored, the dissociation of the salt into ions being taken as complete. Starting with the point at pH 7 on the anæsthetic curve for novocain, the amount of base in the limiting concentration may be calculated. Then the molar concentrations at pH 5, 6, 7 and 8, which contain the same amount of base, can be calculated (see Table III and Fig. 4). The curve is practically a straight line at 45° to the axis, but the slope becomes less between 8 and 9, where [BOH] becomes a large fraction of C. Similar curves drawn parallel to this, but separated by horizontal distances equal to the differences of pK, would represent the theoretical log C/pH curves for the same concentration of base as novocain for the other anæsthetics.

TABLE III.—*Values of the Molecular Concentrations of Novocaine at Different pHs, containing the Amount of Base Necessary to Produce Anæsthesia at a pH of* 7. (0·00013.)

pH.	B.	C (B BOH).	Log. C.
5	0·65554	0·656	$\bar{1}$·8167
6	0·065554	0·657	$\bar{2}$·818
7	0·0065554	0·0668	$\bar{3}$·825
8	0·00065554	0·0078	$\bar{4}$·894
9	0·000065554	0·00019	$\bar{4}$·287

In the first place the slope of the observed anæsthetic curves is not so great as the slope of the theoretical curve for solutions containing equal concentrations of undissociated base.

There are four possibilities which might explain the discrepancy:

(*a*) The effect of the reaction may be directly on the tissue, rendering it more permeable to base, ion and salt alike. The linear relationship between the pH and log C would then only be a striking but accidental coincidence. For this explanation to be valid the increase in permeability with increase in pH for each component of the solution, ion, base and undissociated salt must be exactly the same, which seems an unlikely coincidence when we consider the very different solubilities of each component in fat solvents.

In addition to these arguments, if we take a substance, dissociation of which does not take place at the reactions worked with, namely, benzyl alcohol, no alteration in activity is produced by variations in reaction. The minimal concentration of benzyl alcohol for anæsthesia of the cornea is 1–2%, whether the pH is 6, 7 or 8; certainly no difference occurs of the order observed with the anæsthetic bases. The other possibilities to explain the discrepancy in Fig. 4 all involve the assumption that the base is the most active constituent, and they are:

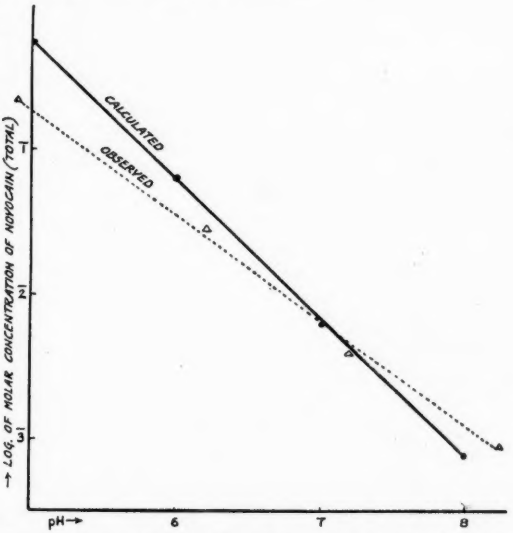

FIG. 4.—Curves showing the relation between the observed and calculated anæsthetic limits for novocain.

(*b*) The ion has an activity which is a small fraction of that of the base.

(*c*) The undissociated salt of the local anæsthetic has anæsthetic activity.

(*d*) The discrepancy is due to the actual pH in the tissues around the nerve-fibre differing from the pH of the applied solution, being less alkaline on the alkaline side of pH 7 and less acid on the acid side of pH 7. If this effect increases with increased distance from neutrality, the result will be similar to that observed. The limiting concentration will be under-estimated on the acid side and over-estimated on the alkaline side, since when the solution applied is on the side of pH 7 there will be a larger proportion of base set free, the solution diffusing into the less acid corneal epithelium, and on the alkaline side a smaller proportion.

Explanation (*b*) is, we think, eliminated by the following argument: Let the anæsthetic activity of the ion [B] be $\frac{1}{r}$ times the anæsthetic activity of

the base [BOH]. Then if the ion could be obtained free from base, the concentration of ion necessary to produce anæsthesia would be r times the concentration of base necessary. The anæsthetic activity of a mixture of base of concentration $[BOH_x]$ and ion of concentration $[B_x]$ would be equivalent to a concentration of base of $[BOH_x + \frac{1}{r} B_x]$. Then in two solutions of the salt of equal anæsthetic activity at different pH's x and y—

$$[BOH_x] + \frac{1}{r}[B_x] = [BOH_y] + \frac{1}{r}[B_y],$$

where $[BOH_x]$ is the concentration of base at pH_x,
" $[B_x]$ " " ion " pH_x,
" $[BOH_y]$ " " base " pH_y,
" $[B_y]$ " " ion " pH_y.

$$\therefore \frac{1}{r}\{[B_x]-[B_y]\}=[BOH_y]-[BOH_x],$$

$$\therefore r = \frac{[B_x]-[B_y]}{[BOH_y]-[BOH_x]}.$$

In the solution just strong enough to cause anæsthesia at pH 5, there is 0·0000367 M base and 0·1884 M ion present, calculated from equations 1 and 2, and the value of r obtained by substitution in equation is approximately 4000. Whereas if the concentrations are worked out for pH 7 and 8, the value of r is only 5·1, and the curve connecting log C and pH, if r had been constant, would therefore have had a pronounced convexity towards the abscissæ between pH 5 and pH 8. Since it is unlikely that there would be such a large change in the value of r with alteration in pH, the conclusion seems inevitable that the ion has no anæsthetic activity.

The explanation (*c*) of the discrepancy by the possession of anæsthetic activity by the undissociated salt is eliminated by a similar argument, which will show that this would also make the relation between log C and pH a curved and not a straight line.

We are therefore left with explanation (*d*), namely, that the reaction to which the nerve-fibre is exposed is nearer neutrality than that of the applied solution; so that the nerve-ending will be exposed to a smaller base concentration on the alkaline side and larger on the acid side, than that which is calculated from the observed pH and limiting concentration.

That variations of reaction do take place in the corneal tissues on application of acid on alkaline fluids to the eye can be demonstrated in several ways:

1. If a 1% solution of neutral red is applied to the cornea, changes in colour of the stained corneal epithelium can be demonstrated when buffered solutions of various pH's are applied to the eye.

2. The reaction of the aqueous humour in a pithed cat becomes more alkaline when alkaline buffer solution is applied to the cornea for a period of 10 minutes.

3. If a 1% solution of cocaine hydrochloride brought to a pH of 3 to 4 with HCl is applied to the eye, as long as the instillation is continued, even for a prolonged period, no anæsthesia develops; but within half a minute of the

cessation of instillation complete anæsthesia develops, and persists for many minutes. The explanation we offer of this observation is that although base diffuses into the cornea during the instillation of the acid solution, the concentration is not sufficient to induce anæsthesia at the acid reaction set up in the cornea. Some of the hydrochloride itself diffuses in. When the instillation ceases, the reaction slowly returns to normal, and the salt which has diffused in sets free more base, owing to the alteration of reaction.

4. The changes in reaction are also shown by the following experiment: A solution of 0·03% benzylbenzoylecgonine hydrochloride in saline, this solution having a pH of 6·43, was instilled into both conjunctival sacs of a rabbit. After 10 minutes' instillation there was no anæsthesia, but almost complete anæsthesia of both corneas developed in about 30 seconds after instillation had ceased. The right eye was then washed with a buffer solution of pH 4, and the left eye with a buffer solution of pH 8. The anæsthesia in the right eye entirely disappeared, while that in the left eye became complete.

These last two experiments show that there is a constant tendency for the reaction of the corneal tissue to return to normal. In consequence, the reaction in the tissue at any time is the result of a balance between the influx of buffers from the applied solution and from the surrounding tissue. We have no information as to the shape of the titration curve of the tissues, but, as was pointed out to us by Dr. Gaddum, if it approximates to a straight line, as it is probable that it does over the reactions studied, then the discrepancy between tissue pH and pH of the applied solution would be proportional to the difference of the pH of the applied solution from 7, or whatever the normal pH of the cornea is.

We think that in this way we can explain the observation of Regnier, that the addition of alkali to a solution of cocaine base in water enhances its anæsthetic power. The concentration of cocaine base necessary to produce anæsthesia is very low and has practically no buffering action. The reaction of the superficial corneal tissues will therefore be little displaced from neutrality, and some of the cocaine will therefore combine to form a salt. On the addition of alkali to the base, the reaction of the corneal epithelium will become definitely shifted towards the alkaline side, and less cocaine will combine with the anions of the tissue and a higher concentration of base will be in contact with the nerve-fibre.

The figures for conessine have not so far been discussed, but they corroborate the theory that the base is the only active fraction in an interesting manner. The first part of the curve is practically a straight line. Conessine is diacidic, and the theoretical curve can be developed in the same manner as for the mono-acidic bases.

$$K_2\,[B\,(OH)_2] = [B\,OH] \times [OH]$$
$$K_1\,[B\,OH] = [B''] \times [OH]$$
$$\text{Therefore } [B''] - \frac{K_1K_2\,[B\,(OH)_2]}{[(OH)]^2} = K_wK_1K_2\,[B\,(OH)_2]\,[H]^2$$
$$\therefore \text{ Log } [B''] = \log K_1K_2K_w + \log\,[B\,(OH)_2] - 2\ \text{pH}.$$

For small concentrations of [BOH] and [B(OH)$_2$], [B″] may be taken as equal to the total concentration of alkaloid, and the slope of the first part of

the anæsthetic curve for conessine should be twice that of the anæsthetic curve for the monacidic bases, if the active component of conessine solution is the undissociated base B $(OH)_2$. This is approximately the case, as will be seen from Fig. 2. The straight line part of the curve extends to pH 7. In order to obtain pH 7·2 in a solution of conessine hydrochloride, it is necessary to add only 2% of the total quantity of soda which is required to set free the whole of the base as B $(OH)_2$. Above this pH the slope of the curve becomes less owing to the relation between B″ and the total alkaloid concentration becoming appreciably less than one. We were unable to obtain values for K_2 for conessine, as precipitation of base occurred in M/300 solution at a pH of 9·1, but pK_1 is approximately 4·6.

The order of the activities of the various substances tried is not the same as the order of their pKs. The most easily dissociated, stovaine, with a pK of 6·14, is not by any means the most active. In Table IV the order of activities is given, in which allowance is made for the difference in dissociation, and the amount of base present in the limiting concentrations at pH 7 is calculated in every case. pH7 is chosen, because the reaction in or around the nerve-fibres is probably near that of the applied solution when the latter is neutral.

TABLE IV.—*The Molecular Concentrations of Free Base Contained in the Highest Dilution Producing Anæsthesia at pH 7.*

Anæsthetic.	C.	BOH.
β-Eucaine	0·0000324	0·00000308
B.B.E.	0·000168	0·00000783
Cocaine	0·000708	0·0000331
Novocain	0·00668	0·000128
Stovain	0·00133	0·000162

These figures then represent the relative anæsthetic activity, what may be called the molecular anæsthetic power, of the bases of the different substances tried. The fact that the bases are so active suggested to Gros that their solubility in oily media may "explain" their action. We tried to determine the partition coefficients of the substances between ether and water. The various experimental difficulties encountered destroyed our confidence in the results obtained, but the figures at any rate gave no indication of differences of partition coefficients of the magnitudes necessary to account for the wide differences in Table IV.

There are therefore two methods of attack for the synthesis of new local anæsthetics. One method is to diminish the basic dissociation constant, and render it possible to obtain a higher concentration of base of the same molecular anæsthetic power, and the other method is to produce structural changes in the molecule, which, while not necessarily diminishing the dissociation constant, might confer increased molecular anæsthetic power on the substance.

It is of course difficult to predict what effect a given structural change will produce on the dissociation constant, and impossible to make any sort of confident prediction of the effect of a structural change on the physiological

similarity of such dissimilar substances as novocain and cocaine. Changes in both will generally result from any structural change. But Gray's attempt to augment the physiological activity of cocaine by the substitution of the benzyl and phenyl ethyl group, which were shown by Macht (1918) and Hjort and Eagan (1919) to have some anæsthetic action of their own, has resulted in two compounds for which the molecular anæsthetic value is considerably higher than that for cocaine, while in the case of the benzyl compound the dissociation constant is not appreciably altered. One interesting point which comes out is that the anæsthetic action of β-eucaine, when reckoned as concentration of free base necessary to produce the effect, is even greater than that of cocaine.

CONCLUSIONS.

(1) The effect of a series of local anæsthetics on the rabbit's cornea at different hydrogen ion concentrations is given.

(2) Plotting the logarithms of the concentration of the minimal effective concentrations against pH is shown to give lines which for all the mono-acidic local anæsthetics are straight and parallel between pH 5 and pH 8.

(3) It is shown that this relationship is consistent with the view that the only active constituent of a solution of local anæsthetic is the free base and not the ion or the undissociated salt.

(4) The relationship between the anæsthetic action and pH for a diacidic base, conessine, is shown to confirm this view.

(5) Further evidence that the influence of pH is due to dissociation of base is drawn from the fact that variations of pH have no influence on the anæsthetic activity of benzyl alcohol on the cornea. It is shown that variations in the dissociation of the basic anæsthetics do not account for the whole of the difference between them, and that therefore there must be some other relationship between the actual molecular structure of the base itself and its anæsthetic power.

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A STUDY OF THE PROPERTIES OF PURE BILIRUBIN AND ITS BEHAVIOUR TOWARDS THE VAN DEN BERGH REAGENT.

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Historical.

SINCE the introduction of the van den Bergh reaction into clinical medicine in 1918, great interest has centred around the constitution of the bile-pigments in serum. A great amount of work has been done in an attempt to explain the differing behaviours of sera towards the diazo reagent. Van den Bergh (1918), using the diazo reagent of Ehrlich, found that some specimens of bile gave a colour reaction when treated with the diazo reagent, while others required the addition of alcohol before any colour change could be observed. He came to the conclusion that if the bile contained any bilirubin, the reaction developed without the addition of alcohol. Van den Bergh also found that alcohol was necessary before any colour change developed on the addition of the diazo reagent to a neutral solution in which bilirubin had been dissolved. Extending his work into the examination of blood-serum, he found that a direct colour effect was obtained when the diazo reagent was added to a serum from a case of obstructive jaundice, and a delayed or negative reaction when the diazo reagent was added to a serum from a case of hæmolytic jaundice.

McNee (1922), who as early as 1912 drew attention to the bile-forming capacity of the reticulo-endothelial system, originated a very neat theory to explain these different types of reaction. According to this theory the type of reaction depends entirely on the fact whether the bilirubin has passed through the hepatic polygonal cell or not. If it has done so, it invariably gives a direct reaction, and the indirect reaction from cases of hæmolytic jaundice is explained as an increase of circulatory bilirubin that has not been through the hepatic cells. Van den Bergh fully agrees with NcNee's conception, and says, "I do not know any clinical observation that would not agree with his theory."

Taking this hypothesis as a basis, many workers have worked on bile-pigments, and have brought forward certain facts to establish a difference between the two forms of bilirubin. Van den Bergh (1921) suggested that the form present in the sera of obstructive jaundice is in a free state, while in those cases giving the indirect reaction it is combined with some protein, and that the addition of alcohol, which breaks up the compound, is necessary before a colour change is possible. In support of his theory he cites the fact that bile-pigments are never found in the urine of cases of anhepatogenous jaundice, however deep the jaundice may be. Writing in the *Presse médicale* (1921), van den Bergh states very emphatically that "dynamic icterus" (*i. e.* the non-obstructive jaundice) is always acholuric whatever may be the bilirubin content

of the serum, so long as there is no obstruction to the bile canaliculi. His reason for this is the supposed combined state in which the bilirubin of dynamic icterus exists, in which state it is unable to pass the renal barrier, while the non-combined bilirubin which he finds in mechanical icterus easily traverses the renal barrier. Thus it was thought that the liver denuded the bilirubin of its protein attachment, and enabled it when it re-entered the circulation to react with the diazo reagent immediately. Both McNee and van den Bergh freely admit that the reason for the different behaviour of icteric sera has not been solved, and the latter concludes one of his articles by saying, "The greatest probability is that the influences that are present in mechanical cases of jaundice and also present in bile are not present in cases of anhepatogenous jaundice" (1924).

Van den Bergh (1918) also remarked on the change in the behaviour of direct sera when allowed to stand in air. It is rapidly oxidized to biliverdin, while that form which comes from a hæmolytic serum does not change, or only to a much slighter degree. Another difference in the behaviour of "obstructive" and "hæmolytic" bilirubin noted by van den Bergh was the varying rate of absorption by protein precipitants, the pigment from the hæmolytic serum not being absorbed, while that of an obstructive serum was readily absorbed.

Grunenberg (1922), working on the solubility of the pigments in chloroform, reported that the bilirubin from "obstructive" sera was not soluble in chloroform, while that from a "hæmolytic" serum was readily soluble. Andrewes (1924[2]) later found that if bile were shaken up with chloroform and the chloroform evaporated, a substance could be obtained which, when dissolved in weak alkali, gave only an indirect reaction. He also found "hæmolytic bilirubin" to be soluble, while that of "obstructive cases" was insoluble. Lepehne (1921) came to similar conclusions. Collinson and Fowweather (1926) define their "free bilirubin" (that which gives the indirect reaction) as being soluble, while that called "alkali bilirubin" (giving the direct reaction) is insoluble in chloroform. Andrewes failed to confirm van den Bergh's work on the adsorption on to protein precipitates.

Work was also published by Hoover and Blankenhorn (1916) on the difference in the behaviour of bilirubin towards collodion membranes. "Obstructive" bilirubin dialysed with ease, while that of "hæmolytic" type did not. Other workers (Leschke, 1921; Brulé, Garban and Weissman, 1922) have supported these observations made by Hoover and Blankenhorn.

Collinson and Fowweather (1926) recently published a study of the constitution of bile-pigments in sera, and came to the conclusion that the direct and indirect reaction can be explained by observing whether the bilirubin is combined with ammonia as a salt or existing as free bilirubin in the sera. In cases of indirect reaction they state that the bilirubin is free, while that which has passed through the hepatic cell is in the form of ammonium bilirubinate.

While most workers have maintained that there is a change in the bile-pigments in the serum in the different types of jaundice, Adler and Strauss (1922) have attributed the various changes to the media—"direct or indirect occurs according to the hydrated or dehydrated state of the proteins." This

they give as the explanation why certain salts enhance the colour of the reaction. "The greater the dehydrating effect of the salt, the more rapid the diazo coupling." The salts that have the property of enhancing the colour are ammonium sulphate, caffein sodium salicylate, urea, etc. Thannhauser and Andersen (1921) thought that cholesterol or bile acids might accelerate the reaction, but experiments did not confirm this view.

It can be seen from this brief *résumé* of the literature that there is much disagreement regarding the constitution of the bile-pigments in serum. The present writers have investigated the problem, and have only been able to confirm a few of the earlier observations.

Summing up the literature it can be gathered that the main factors to be discussed are van den Bergh's protein-combining theory, Adler and Strauss's change in the media, with Thannhauser and Andersen's increase of cholesterol and bile-salts, and Collinson and Fowweather's ammonia combination.

Van den Bergh's Protein Combining Theory.

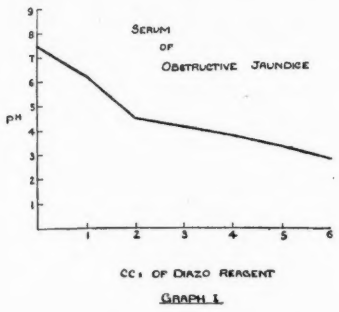
Andrewes (1924[2]) investigated this question, and found no evidence to support the statement that bilirubin giving the indirect reaction is combined with a protein compound. He found that neutral solutions of bilirubin which gave the indirect reaction were quite free from protein, and in his paper Andrewes states that the bilirubin obtained from old gall-stones behaves in the reaction towards the diazo reagent as "hæmolytic bilirubin." We have evidence against this, as bilirubin behaves in a constant manner towards the diazo reagent quite irrespective of its source—whether it has been obtained from gall-stones or direct from a serum of obstructive jaundice. Again the direct reaction is by no means always obtained with specimens of bile from the gall-bladder, whereas if it is true that the liver frees the bilirubin from its protein attachment, then the bile obtained in the gall-bladder should always give the direct reaction. Our experiments showed that this was not so, the addition of alcohol being frequently necessary. We felt therefore that the investigation of the action of the diazo reagent on crystalline bilirubin would probably clear up some of these points.

The Van den Bergh Reaction on Pure Bilirubin Solution.

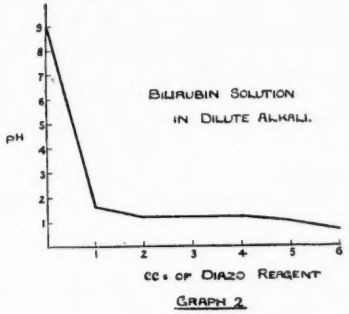
Bilirubin was obtained in a pure state from gall-stones. It is a bright yellow powder insoluble in water and in alcohol, but soluble in slightly alkaline solution. It is readily soluble in chloroform.

Many workers have investigated pure bilirubin solution in its behaviour towards the diazo reagent, and have found that unless an abnormally high degree of alkalinity exists in the solution it is not possible to get a direct reaction. Andrewes uses this fact as a reason for calling the bilirubin in pure solution "hæmolytic bilirubin," as it only gives a reaction after the addition of alcohol, and states that "evidence is brought forward to show that it is identical with it." Van den Bergh also failed to get a direct reaction, and assumes therefore that the bilirubin that has been obtained from old gall-stones must be of different composition from that found in the serum and fresh bile. But there is no reason why, under identical conditions, the

bilirubin, however produced, should behave in any different manner when the diazo reagent is added, provided it has not been in solution for some time. The diazo reagent is acid in reaction, its *p*H is below 1, and the addition of this reagent produces quite different results on the *p*H of serum and of solutions of bilirubin. The effect of adding varying quantities of the diazo reagent to 6 c.c. of serum from a case of obstructive jaundice is shown in



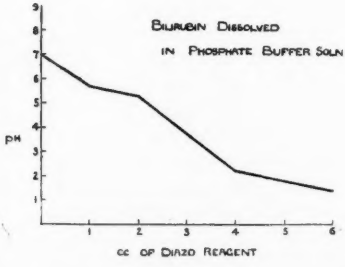
GRAPH 1.



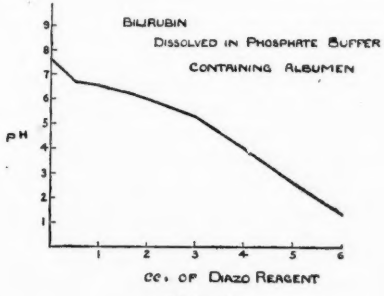
GRAPH 2.

Graph 1. There is a gradual fall in the *p*H with the addition of increasing quantities of diazo reagent from the initial *p*H of 7·8 to 2·8. The van den Bergh reaction is direct through the whole range down to 2·8.

But quite a different picture is observed when the diazo reagent is added in like quantities to 6 c.c. of a pure bilirubin solution which has been prepared



GRAPH 3.



GRAPH 4.

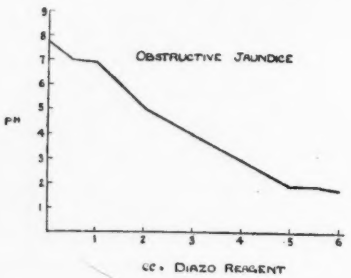
by suspending some of the powder in distilled water, and dissolving by gentle heat and the addition drop by drop of dilute alkali. The initial *p*H was 9, and after the addition of only 1 c.c. of diazo reagent the *p*H had fallen to 1·8, compared with the corresponding value of 6·3 in the serum.

Graph 2 is totally different from that of serum, and a low *p*H is obtained so rapidly that even small amounts of diazo reagent lower the *p*H so much that coupling fails to occur and the van den Bergh reaction cannot be obtained.

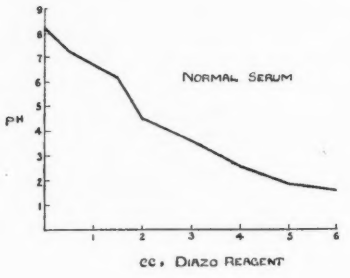
The buffering properties of the blood kept the *p*H of the serum at a high figure, making coupling possible, and the absence of this property fully explains the absence of the direct reaction in a pure bilirubin solution if it is not buffered.

It was then thought advisable to dissolve bilirubin in a buffered solution. This was done with phosphate buffer, and again with phosphate buffer in which some serum albumin was dissolved. Both are shown in Graphs 3 and 4, and the gradual fall in the *p*H resembles that of serum, and in this solution the van den Bergh reaction is now direct on the addition of 1–4 c.c. of diazo reagent.

These charts show that bilirubin has no different reaction whether produced from gall-stones or obtained from an obstructive jaundice case, and that it cannot be regarded as identical with "hæmolytic bilirubin," as Andrewes suggested. The reason that the direct reaction is not obtained in a pure bilirubin solution is merely a question of hydrogen ion concentration. It is



GRAPH 5



GRAPH 6

seen in Graph 2 that the addition of even one-sixth quantity of the diazo reagent to a bilirubin solution immediately lowers the *p*H to a value at which the reaction cannot be obtained, but once a solution of bilirubin is made up resembling blood-serum the van den Bergh reaction becomes direct.

Since the free circulation of bile-salts and cholesterol is impeded in mechanical jaundice, we thought it would be advantageous to investigate and compare the buffering properties of the serum of a clear-cut mechanical jaundice and that of a normal serum by adding like quantities of the diazo reagent to each. Graphs 5 and 6 show the effect. No appreciable difference is obtained, and therefore we agree with Thannhauser and Andersen that the increased bile-salt and cholesterol content cannot be brought forward as explaining the prompt reactions of obstructive cases. The addition of bile-salts or cholesterol did not affect the diazo reaction of a bilirubin solution.

Collinson and Fowweather's ammonia combination was also investigated, and it was found that the ammonia only enhanced the rate of coupling by increasing the alkalinity of the solution and not apparently in any specific manner. When regarded in this light it was not surprising to find that it did not in any way act so efficiently in this direction as a phosphate buffer solution.

The Chloroform Solubility of Bilirubin.

Pure bilirubin is easily soluble in chloroform, but when gall-bladder bile is shaken up with chloroform the extraction takes some time. If this chloroform extraction, which is now of the "obstructive" type as it has passed through the hepatic cell, be gently heated to evaporate the chloroform and the residue be dissolved in a phosphate buffer solution of *p*H 7·5, the direct reaction is obtained immediately.

Again, chloroform readily dissolves the bilirubin from the dry residue of gall-bladder bile that has been heated to dryness over a water-bath. This residue is also soluble in phosphate buffer solution, and gives an immediate colour reaction with the diazo reagent. It can be seen, therefore, that chloroform readily extracts bilirubin from gall-bladder bile, and this bile gives the direct reaction.

Quite a different state occurs when the dried residue of a serum from an obstructive jaundice is shaken up with chloroform. The pigment does not enter into solution. Furthermore, chloroform does not extract any of the pigment from a watery solution of this dried jaundiced serum.

Again, if crystalline pure bilirubin, which we know to be easily soluble in chloroform, be dissolved in ordinary serum and then filtered to give a clear icteric serum (one that now gives the direct van den Bergh reaction), and if this serum is now dried in a desiccator, the yellow residue is no longer soluble in chloroform, but easily soluble in water. Therefore it can be concluded that the chloroform solubility property of bilirubin can hardly be brought forward to distinguish between obstructive and hæmolytic bilirubin. Is it not entirely due to the physical characteristics of the serum?

Dialysing Properties.

We have already referred to the work of Hoover and Blankenhorn on the dialysing properties of the bile-pigments. Some experiments were conducted along these lines, and it was found that if bilirubin was dissolved in normal serum, no dialysis occurred, while if the bilirubin was dissolved in a phosphate buffer solution, dialysis rapidly occurred, yet in both solutions the van den Bergh reaction was direct. Gall-bladder bile dialyses almost as rapidly as a phosphate solution of bilirubin. The pigment from a serum giving the direct van den Bergh reaction did not dialyse.

Again, as in the chloroform solubility, it can be seen that it is the media that controls the dialysing properties of bilirubin, and that this behaviour can hardly be used as evidence in support of two forms of bilirubin.

Evidence in Favour of an Oxidative Change.

It is a well-known fact how the gall-bladder bile varies in colour in post-mortem cases. In some it is light yellow and in others deep green in colour. Again it is only necessary to call to mind the various changes in colour that a case of carcinoma of the pancreas, which is deeply jaundiced, undergoes; how at the onset the icterus is light yellow, and later how it has changed not only in intensity, but also there is a decided change in colour—the deep olive green

of the terminal stage of obstructive jaundice. A similar change takes place in the common bruise, when the hæmoglobin is being broken down by the tissue-cells and the surrounding tissue has been tinged a light yellow, which is later transformed into a deep green. These changes are analogous to the change in colour occurring in the pure bilirubin solution when allowed to stand for a number of hours, when the light yellow colour has been entirely replaced by a dark green. This change may explain the diverse and contradictory results obtained when the diazo reagent is added to a serum containing a pathological amount of bile-pigments.

One of the first experiments undertaken was the preparation of solutions of varying strength of bilirubin, ranging from one of a content of 1 unit to one of a content of 200 units. When the diazo reagent was added no reaction could be obtained, and it was remembered that the solution had been allowed to stand overnight and the decided change in colour had been overlooked. The question then arose as to whether this change was of an oxidative nature.

One of van den Bergh's reasons for asserting a change in the bilirubin itself—a change in constitution—is the different rate of oxidation, the "obstructive" type being easily oxidized, while the "hæmolytic" type is not oxidized. It is not clear from the account how the oxidation rate was measured. If two sera are taken—one which gives the direct reaction and the other only giving the indirect reaction—and both allowed to stand in air in open vessels for a period of forty-eight hours, the direct reaction will no longer be present in the first, but the indirect will be obtained precisely as before in the second. Andrewes (1924[1]) noted that sera that gave the direct reaction if allowed to stand would eventually give a delayed reaction similar to that of a hæmolytic jaundice serum. He also noted that the rate of the diazo reaction in hæmolytic sera was the same when the sera had been left standing for several days.

A review of these facts suggested that, on standing in air, bilirubin solutions became converted to oxidation products such as biliverdin, and that the delayed and indirect reactions might possibly be due to these oxidation products. It has been stated elsewhere by van den Bergh himself that biliverdin will not give a reaction with the diazo reagent either alone or after the addition of alcohol, but our observations do not confirm this statement. The following experiments bring forward evidence in favour of an oxidative change.

Bilirubin was dissolved in a phosphate buffer solution and a few drops of hydrogen peroxide added to the solution. On shaking, a change in colour was observed, and the van den Bergh reaction, while direct at first, now became indirect.

Again, serum which came from a case of obstructive jaundice and which gave a marked immediate direct reaction, was treated with hydrogen peroxide in a similar fashion, shaken up and then diazotized. The direct reaction was no longer obtained, but on adding alcohol the colour reaction was obvious—a direct transformed into an indirect. Oxidation was demonstrated in a pure solution and in serum.

Another experiment on these lines consisted in replacing the hydrogen peroxide with oxygenated red cells. A bilirubin solution which gave the direct reaction was shaken up with washed red cells, centrifuged and treated

with the diazo reagent. A direct reaction was no longer obtained, and the positive change only seen after the addition of alcohol.

The next step was the production of biliverdin. A strong solution of bilirubin was allowed to stand for two days, when the colour had completely changed to a dark green, a solution which gave no further direct action with the van den Bergh reagent. The pigment was precipitated from solution with hydrochloric acid and then washed with chloroform, which dissolved out any unchanged bilirubin. The biliverdin remaining was extracted with alcohol, in which it is very easily soluble, giving an emerald green solution. This alcoholic solution gives an immediate change with the diazo reagent. On evaporating the alcohol, biliverdin remained in a comparative state of purity as a dark green amorphous mass, which is much more soluble in dilute alkali than bilirubin, but insoluble in water. The alkali solution of biliverdin gives no reaction with the diazo reagent, except a purple colour of fair intensity only after twelve hours' standing. There is no change at all for two hours. While this direct reaction is negative, alcohol added to the solution gives an immediate colour effect.

Bile-Duct Ligation Experiments.

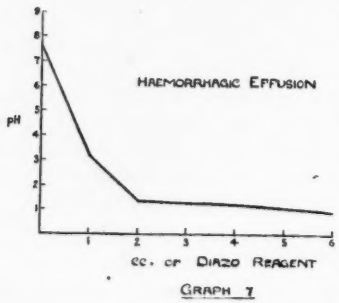
It was thought that the study of the bile-pigments in the serum of a clear-cut mechanical obstruction would be profitable, and the bile-duct was therefore ligated in a rabbit under ether anæsthesia. Twenty hours after the operation the diazo reaction on the serum was negative in the direct sense, and only a faint purple hue developed after fifteen minutes' standing. The cholesterol was also estimated and found to be 94 mgm. per cent. At the end of twenty-five hours the colour developed in three minutes, but was still very faint and could not in any way be regarded as a direct reaction. Forty hours after the operation the serum gave a purple colour with the diazo reagent in one minute, but still an absence of a prompt coloration. The Fouchet test was at this time positive—a finding which dispelled any doubts as to the correctness of the ligation. The cholesterol value was now 110 mgm. per cent. Sixty-six hours after the ligation the van den Bergh reaction was beyond doubt a true direct, and the cholesterol figure had increased to 140 mgm. per cent. At this point the sclerotics were jaundiced, bile was present in huge quantities in the urine, and the fæces were pale.

Similar results were obtained by Lepehne (1921). The pigment that is present in the circulation should, according to the existing theory, give a direct diazo reaction. It is difficult to explain our results on this basis. It is of course possible that a sudden obstruction is enough to damage and inhibit all cellular activity of the liver, and the passage of the pigment through the polygonal cell is prevented. But the delayed and indirect reactions are later replaced by the direct.

The Behaviour of Hæmorrhagic Effusions to the van den Bergh Reagent.

It is well known that old hæmorrhagic effusions, such as pleural and peritoneal, give a positive indirect reaction, and no record has ever appeared of one giving the direct van den Bergh reaction. This has been explained by the fact that the bilirubin manufactured at this seat has not traversed the

hepatic cells. It could not be that oxidation played any great part here, so that the change to biliverdin or any other intermediate product could hardly take place. Fortunately a case of carcinoma of ovaries with a hæmorrhagic effusion into the peritoneal cavity presented itself, and the reddish-yellow exudate was obtained. This gave no direct van den Bergh reaction, but only a faint indirect reaction. The effect of adding the diazo reagent to this effusion was studied, and Graph 7 shows the abrupt drop in *p*H, almost similar to that of a solution of bilirubin which possesses no buffer salt.



GRAPH 7.

If in this case a half volume of diazo reagent had been added to one volume of the effusion the *p*H of the mixture would be as low as 1·2, and no diazo coupling can occur here.

It would appear that the indirect reaction given by hæmorrhagic fluids can be explained by their deficiency in buffering power, but it is obviously impossible to give a final answer to this problem until many specimens have been examined.

The Action of Alcohol.

The action of alcohol is not at all clear except that it can possibly be explained by the easy solubility of biliverdin and possibly some intermediate products in the alcohol. It was found that methyl, ethyl and propyl alcohol gave positive reactions, while negative results were obtained when butyl and amyl alcohols were used. Acetone acts as well as ethyl alcohol.

The above experiments suggest the following:

That the behaviour of bilirubin towards the diazo reagent is constant, and that changes are either due to alteration in the *p*H of the solution or to an oxidative change occurring in the pigment.

That the chloroform solubility and dialysis properties vary with the solvent.

That clinical and experimental evidence supports the view that bilirubin can be oxidized to biliverdin in the circulation, which pigment only gives the indirect reaction.

It is felt that this view explains in a like manner the varying results obtained with the van den Bergh reaction. It does not in any way detract from the value of the reaction, but it seems to shed light on those not infrequent cases that do not fall in with the clinical expectations.

It is offered as an explanation that in obstructive jaundice there is a constant absorption of bilirubin into the circulation from the distended bile-channels—channels which are more or less long sealed tubes—in which oxidation cannot freely occur. It is interesting to record that as early as 1889 Haycraft and Scofield observed the various changes that occurred in the constitution of the bile-pigments found in the gall-bladder. They came to

the conclusion that reduction of biliverdin into bilirubin was a common phenomenon, as bile in the middle of the gall-bladder was green and the bile near the bladder-wall was orange-brown. In consequence, bilirubin is present in just sufficient amount almost constantly in the circulation to give a direct diazo reaction. In the early stages of obstruction and when the obstruction is being relieved it is well known clinically how the van den Bergh reaction changes. In the early and late stages (when the icterus has been relieved) it may vary from a delayed to a negative direct. The icterus is not very deep, and oxidation may have been sufficient to transform the bilirubin into biliverdin. This change is well seen in the superficial tissues—a change which can take place quite as readily in the circulation.

Van den Bergh, in his work, says that he frequently came across a direct diazo reaction in some cases of icterus due to terminal heart failure, and after a careful search no anatomical changes could account for any obstruction. Eppinger's biliary thrombi were not observed. The direct reaction here can be explained possibly by the lack of oxidation, bilirubin not being changed as readily into biliverdin as in those cases with an intact circulation.

The daily variations obtained in cases of catarrhal jaundice can be accounted for if one regards as the criterion of a direct reaction the presence of bilirubin in the circulation.

It is a pleasure to thank Mr. W. Lawson for the great trouble he took to prepare samples of pure crystalline bilirubin.

Dr. J. W. McNee very kindly supplied us with some of the literature and read through the paper prior to publication. He raised the question of the reaction of hæmorrhagic effusions, which we have now dealt with in the text. It is hoped that the experiments advanced answer this important query, but of course Dr. McNee is in no way responsible for any of the conclusions drawn by ourselves.

We are also indebted to Prof. McIntosh for reading through the paper.

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THE EFFECT OF STREPTOCOCCAL TOXIN ON THE BACTERICIDAL POWER OF HUMAN BLOOD.*

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THE Dicks' experiments on the filterable toxin of *Streptococcus scarlatinæ* have been followed by a large amount of work on the toxins of streptococci from scarlatina and other diseases. Eagles (1926), who tested the toxigenic properties of hæmolytic streptococci from scarlatina, erysipelas, puerperal fever and other sources, found that filtrates from all the strains of *Streptococcus scarlatinæ* examined by him gave a positive Dick test in a dilution of 1 in 1000. His results with filtrates from hæmolytic streptococci derived from other sources were less uniform: some of the filtrates caused a positive Dick reaction in a dilution of 1 in 1000, some failed to cause a positive reaction unless the filtrate was used in a dilution of 1 in 10, and filtrates were prepared from other strains which did not give a positive Dick reaction when the dilution was reduced to 1 in 10.

The experiments described in this paper were carried out to determine whether streptococcal filtrates which give a positive Dick reaction are also toxic to human blood *in vitro*, and conversely whether filtrates which do not cause any skin reaction are correspondingly inactive when tested on blood. It will be shown that streptococcal toxin has some poisonous action on blood which can be neutralized by antitoxin, but this poisoning effect is so feeble and the toxin must be used in such a high concentration before any evidence of its action on blood can be demonstrated that any attempt to test the strength of a toxin by this method would be useless.

METHOD OF TESTING THE ACTION OF STREPTOCOCCAL TOXIN ON HUMAN BLOOD.

In the preliminary experiments the slide-cell technique (Wright, Colebrook and Storer, 1923) was used for testing the effect of streptococcal toxin on human blood. The bactericidal actions of normal blood and of blood to which toxin had been added were compared, and any change in the antibacterial action of the blood due to the addition of the toxin was noted. It is difficult to provide such experiments with adequate controls, as the addition of any foreign substance to human blood is liable to alter its bactericidal power. Fleming (1924) has shown that antiseptics profoundly affect the bactericidal

* The expense of this research was partly defrayed by a grant from the Medical Research Council.

power of human blood, and it is therefore imperative to use only toxins and sera which are free from any antiseptic. Dr. O'Brien, of the Wellcome Research Laboratories, has kindly supplied me with Dochez toxin and antitoxin free from antiseptics.

It can be shown by experiments on the bactericidal power of blood that normal broth is toxic to leucocytes, and this can also be demonstrated by observing the power of broth to prevent emigration of leucocytes *in vitro*. It is therefore necessary to guard against confusing the poisonous effect of broth with the action of a toxin. The Dick test, which consists of the intradermal injection of toxin into the human skin, is controlled by making a similar injection of toxin which has been heated at 100° C. for one hour, and this control was adopted in my first experiments on the action of the toxin on blood. My own blood was used in all the experiments, and after the conclusion of the work an intradermal injection of Dochez toxin into my skin was followed by a positive Dick reaction, showing that my blood did not contain antitoxin.

Table I shows the action of Dochez toxin on the bactericidal power of my blood in slide-cells. Four dilutions of staphylococci were used, and the table gives, in the first column, the number of cocci implanted in the blood in each dilution; in the second column, the number of cocci which survived in normal defibrinated blood; in the third column, the number of cocci which survived when Dochez toxin was added to the blood in a dilution of 1 in 5; and in the fourth column, the number of cocci which survived when heated Dochez toxin was added to the blood. The toxin caused a reduction in the bactericidal power of the blood, and the heated toxin was less poisonous than the unheated toxin.

TABLE I.—*Showing the Effect of Dochez Toxin on the Bactericidal Power of Human Blood.*

Number of staphylococci planted in 50 c.mm. of blood.*	Number of colonies which developed in 50 c.mm. of blood.	Number of colonies which developed in 50 c.mm. of each of the following mixtures:	
		Blood 4 parts + toxin 1 part.	Blood 4 parts + heated toxin 1 part.
168	23	77	37
84	13	37	21
42	1	14	8
21	2	6	2

This experiment was controlled by similar experiments in which normal broth was substituted for the toxin and normal broth which had been heated for one hour at 100° C. was substituted for the heated toxin. It appears from these control experiments that broth which has been heated at 100° C. for an hour, or has been re-autoclaved, is less poisonous to blood than fresh broth which has been heated as little as possible to ensure sterilization.

TABLE II.—*Gives the Result of a Control Experiment with Heated and Unheated Normal Broth.*

Number of staphylococci planted in 50 c.mm. of blood.*	Number of colonies which developed in 50 c.mm. of blood.	Number of colonies which developed in 50 c.mm. of each of the following mixtures:	
		Blood 4 parts + broth 1 part.	Blood 4 parts + heated broth 1 part.
248	32	49	30
124	8	23	18
62	6	19	5
31	2	7	5

The broth which had been subjected to additional heating was less poisonous than the normal broth, and the heated toxin used in the Dick test to control the action of toxin was therefore discarded as a control in these experiments. The control used in the following experiments was broth of the same batch as that used in the preparation of the toxin, and this is not an entirely satisfactory control owing to the alteration in pH due to the growth of streptococci. This objection does not, however, appear to be important, as no difference is found between the toxic action on blood of a broth control and of a filtrate from a non-toxic strain of streptococcus, although the pH of the latter has been altered by the growth of the streptococci. In the following experiments staphylococcus was discarded as the test organism, and in each case the streptococci from which the toxin was prepared were used in its stead. As the slide-cell method is not suitable for testing the bactericidal power of blood on hæmolytic streptococci, a technique previously described (1927) was used in which the cocci are incubated with blood on a mixing machine for three hours, and the number of surviving cocci is then found by plating out a portion of the blood in an agar plate.

THE ACTION OF DOCHEZ TOXIN ON BLOOD *IN VITRO*.

The Dochez toxin and antitoxin used in these experiments were free from antiseptics; the controls were normal broth of the same batch as that used in the preparation of the toxin and normal horse-serum. The intradermal injection of 0·2 c.c. of this toxin in a dilution of 1 in 1000 caused a positive Dick reaction. 1 part of undiluted toxin was added to 39 parts of defibrinated blood, and 1 part of undiluted antitoxin was added to 19 parts of blood. A 16-hour broth culture of Dochez streptococcus was used as the test organism in a dilution of 1 in 1000. The following figures show that the toxin is definitely more poisonous to blood than the control broth, and that by damaging the bactericidal power of the blood it allows a larger number of streptococci to survive.

* The figures in the upper part of this column were calculated from a count of the implants used in the lower dilutions.

Number of streptococci	implanted in 50 c.mm. of blood	. .	8400
,, ,,	which survived in 50 c.mm. of blood	.	61
,, ,,	which survived in 50 c.mm. of blood + control broth in a dilution of 1 in 40	.	78
,, ,,	which survived in 50 c.mm. of blood + toxin in a dilution of 1 in 40	. .	319

The following figures show the effect of Dochez antitoxin on the bactericidal power of blood when Dochez streptococcus is used as the test organism. Unfortunately my blood is sensitive to horse-serum, but in spite of the poisonous action of normal horse-serum, the antitoxin caused considerable improvement in the bactericidal action of the blood.

The mechanism of the beneficial action of Dochez antitoxin on the bactericidal power of blood for the homologous coccus is unknown, but it is probable that some part of this beneficial action is due to the neutralization of the small amount of toxin introduced with the infecting cocci.

Number of streptococci	implanted in 50 c.mm. of blood	. .	8400
,, ,,	which survived in 50 c.mm. of blood	.	61
,, ,,	which survived in 50 c.mm. of blood + control normal horse-serum in a dilution of 1 in 20	. . . . .	161
,, ,,	which survived in 50 c.mm. of blood + antitoxin in a dilution of 1 in 20	. .	21

The addition of mixtures of toxin and antitoxin to the blood with the intention of demonstrating the neutralization of the toxin caused rather indefinite effects. This is not surprising when we consider that each of the foreign substances added to the blood has a poisonous action on the leucocytes, and that we are therefore measuring not only the neutralization of a specific toxin, but also the summation of the effects of two poisonous foreign substances. This difficulty was overcome by substituting human antitoxin for horse antitoxin, and it will be seen later that when human serum is used, the results are much more definite.

The following figures show the results of adding a mixture of toxin and antitoxin to the blood with controls of broth and normal horse-serum. The sera were used in a dilution of 1 in 20 in the blood, and the toxin and control broth in a dilution of 1 in 40.

Number of streptococci	implanted in 50 c.mm. of blood	. .	8400
,, ,,	which survived in 50 c.mm. of blood	.	61
,, ,,	which survived in 50 c.mm. of a mixture of blood + broth control + normal horse-serum control	. . . .	119
,, ,,	which survived in 50 c.mm. of a mixture of blood + toxin + normal horse-serum control	. . . . .	239
,, ,,	which survived in 50 c.mm. of a mixture of blood + broth control + antitoxin	.	49
,, ,,	which survived in 50 c.mm. of a mixture of blood + toxin + antitoxin	. .	131

THE ACTION OF TOXIN DERIVED FROM PUERPURAL HÆMOLYTIC STREPTOCOCCI ON BLOOD *IN VITRO*.

Filtrates were prepared from (4-day) cultures in Douglas's trypsin digest broth of two strains of hæmolytic streptococci which had been isolated by blood culture from cases of puerperal septicæmia. These two strains were examined by Eagles for their power of toxin production, and they were selected for the following experiments because he found that one of them was the most highly toxigenic of all the puerperal strains he examined, while the other did not produce any toxin. The filtrates were tested by the method described above, a culture of the streptococcus from which the filtrate was prepared being used as the test organism. The filtrate derived from the toxigenic strain was toxic to blood, and this effect was neutralized by antitoxin, but the filtrate from the atoxic strain did not alter the bactericidal power of the blood.

1. *Toxigenic Strain.*

One part of the filtrate derived from the toxigenic strain was added to 39 parts of my blood, and the control broth was used in the same proportion. Serum from a patient who had been infected with this streptococcus and had recovered was used as the antitoxin, 1 part of serum being added to 10 parts of blood, and this was controlled by the use of my own serum in the same proportion. The following figures show that the filtrate was more toxic than the control broth and that the patient's serum neutralized this toxic effect:

Number of streptococci	implanted in 50 c.mm. of blood . .	1800
,, ,,	which survived in 50 c.mm. of blood .	33
,, ,,	which survived in 50 c.mm. of a mixture of blood + control broth + normal serum	106
,, ,,	which survived in 50 c.mm. of a mixture of blood + toxin + normal serum .	236
,, ,,	which survived in 50 c.mm. of a mixture of blood + control broth + antitoxin .	110
,, ,,	which survived in 50 c.mm. of a mixture of blood + toxin + antitoxin . .	77

The following figures show the results obtained when the toxin and control broth were diluted 1 in 10 in normal serum before being added to the blood. Apart from this alteration the conditions of the experiment were not changed.

Number of streptococci	implanted in 50 c.mm. of blood . .	1800
,, ,,	which survived in 50 c.mm. of blood .	33
,, ,,	which survived in 50 c.mm. of a mixture of blood + broth control + normal serum	38
,, ,,	which survived in 50 c.mm. of a mixture of blood + toxin + normal serum .	107
,, ,,	which survived in 50 c.mm. of a mixture of blood + broth control + antitoxin .	23
,, ,,	which survived in 50 c.mm. of a mixture of blood + toxin + antitoxin . .	11

It appears from both these sets of figures that the toxin is not only neutralized by the antitoxin, but the toxin-antitoxin mixture has a beneficial action on the bactericidal power of the blood in comparison with the control mixture containing broth and normal serum.

2. *Atoxic Strain.*

We have just seen that a filtrate capable of producing a positive Dick reaction has a toxic effect on blood *in vitro*. The next experiment shows that a similar filtrate from a puerperal strain of hæmolytic streptococcus which does not contain Dick toxin is not poisonous to blood *in vitro*. The following figures show the effect of adding the undiluted filtrate to blood in the same proportions as in the previous experiments. The homologous streptococcus was used as the test organism.

Number of streptococci		implanted in 50 c.mm. of blood .	31,800
,,	,,	which survived in 50 c.mm. of blood	103
,,	,,	which survived in 50 c.mm. of blood + control broth in a dilution of 1 in 40	123
,,	,,	which survived in 50 c.mm. of blood + filtrate in a dilution of 1 in 40	111

This experiment was repeated, using *Streptococcus viridans* as the test organism, with the following result:

Number of streptococci		implanted in 50 c.mm. of blood .	100
,,	,,	which survived in 50 c.mm. of blood	6
,,	,,	which survived in 50 c.mm. of blood + control broth in a dilution of 1 in 40	24
,,	,,	which survived in 50 c.mm. of blood + filtrate in a dilution of 1 in 40	23

Eight different filtrates have been prepared from this atoxic strain of streptococcus by growing it in broth for periods varying from 16 hours to 1 month, but no evidence has been obtained that any of these filtrates have a toxic effect on human blood.

DISCUSSION.

It has been shown that streptococcal filtrates, capable of producing a positive Dick reaction, cause a decrease in the bactericidal power of blood, but that filtrates derived from hæmolytic streptococci, which do not contain any toxin recognizable by the Dick test, are not poisonous to blood. The concentration of toxin required to alter the antibacterial action of blood is very high in comparison with the concentration required to produce a positive Dick reaction, and even when a large quantity of toxin is added to blood the

bactericidal power is only slightly effected. It has been shown in a previous paper (1927) that the power of multiplication of hæmolytic streptococci in human blood is dependent on the virulence of the culture. If a highly virulent culture is used the streptococci multiply freely in the blood, but if an attenuated culture is used the blood is enabled to destroy the cocci. The addition of toxin to blood does not have a sufficiently toxic effect on leucocytes, under the experimental conditions employed, to cause an attenuated culture to multiply freely in human blood, and the fall in bactericidal power due to the addition of toxin to blood is so small that it does not supply a possible alternative method of testing streptococcal toxin.

It has been shown (1926) that the formation of precipitates in bactericidal mixtures will greatly diminish and may totally destroy the bactericidal action of the leucocytes, and it is therefore necessary to discount the effect of precipitate formation in experiments where the power of antitoxin to neutralize the effect of toxin on blood is being investigated. In all the experiments recorded in this paper the toxin-antitoxin mixture did not form any precipitate in the dilutions used. It is frequently noticeable that in the absence of precipitate formation the combined action of antigens and antibodies on the bactericidal power of blood is beneficial, and this phenomenon appears in the experiment on human antitoxin quoted above.

The Dick test can only be carried out in human skin, as the intradermal injection of toxin into the skin of animals is not followed by a positive Dick reaction, but fresh defibrinated horse-blood is affected by streptococcal toxin and antitoxin in the same way as human blood.

CONCLUSIONS.

1. Streptococcal toxin has a slight but definite toxic action on the bactericidal power of blood *in vitro*, and this effect can be neutralized by antitoxin.
2. Streptococcal filtrates, capable of producing a positive Dick reaction, are slightly toxic to blood *in vitro*.
3. Filtrates from hæmolytic streptococci which do not give rise to a positive Dick test are not toxic to blood.

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PIGMENT OBTAINED FROM FÆCES.

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WHEN fæces are subjected to prolonged heating with an alcoholic solution of hydrochloric acid, *e.g.* as in fæcal fat analysis, a brilliant rose-red pigment is obtained (Harrison, 1924). The nature of this pigment has been determined.

Preparation of the Pigment.

Samples of dried fæces are suspended in a 1% solution of concentrated hydrochloric acid in absolute alcohol, and the mixture is evaporated to dryness on the water-bath. This process is repeated 6–9 times, and the solid gradually assumes a pinkish tint. The dried residue is pulverized, extracted exhaustively with ether in a Soxhlet apparatus to remove fatty materials, the residue is freed from ether, and the solid extracted in a Soxhlet with chloroform to remove stercobilin, etc. The solid is then extracted several times with hot dilute aqueous or alcoholic hydrochloric acid, the extracting fluid becoming brilliant rose-red. The pigment is avidly extracted from aqueous solution by amyl alcohol, and the solution in amyl or ethyl alcohol is dried by anhydrous sodium sulphate.

When the solvent is evaporated off *in vacuo* a dark red pasty solid is obtained which is precipitated by the addition of dry ether in the form of dark amorphous flecks.

Properties of the Pigment.

The pigment is soluble in amyl and ethyl alcohol, and dilute mineral acids (except nitric acid, which destroys the colour), but insoluble in ether, benzene and chloroform. The dried amyl alcohol solution of the pigment exhibits an absorption band in the yellow-green portion of the spectrum (about $\lambda = 5600$–5800 Å.U.), and partially purified specimens also display a faint band at $\lambda = 4900$–5100 Å.U., possibly due to the presence of stercobilin, etc. The pigment is rose-red in neutral solution and more scarlet when acid. Changes occur

when alkaline (see later). In anhydrous amyl alcohol the pigment has been kept for over twelve months without apparent decomposition; in dilute aqueous or alcoholic hydrochloric acid, however, the pigment disappears completely in two months, leaving a yellow-brown solution. Although stable to moderate warming, prolonged treatment at 100° results in formation of a yellow-brown solution. Mild oxidizing agents also give rise to a brown colour, and reducing agents also discharge the colour. Attempts to restore the colour by treatment with mild oxidizing agents were unsuccessful, possibly owing to the sensitivity of the pigment to oxidation. Addition of minute amounts of even a weak base (*e.g.* ammonium hydroxide or some tap-waters) to aqueous media containing the pigment gives rise to an evanescent green colour, which immediately disappears, leaving a brown solution from which the pigment could not be recovered by acidification, etc. When weak alkalies are added to anhydrous media containing the pigment the green colour persists for as long as thirty minutes and is of a very clear tint, but appears to possess no definite bands in its absorption spectrum. When acids are added to the green solution the rose-red pigment is regenerated. When allowed to stand the green solution in ethyl alcohol deposits an amorphous brown solid, leaving the supernatant liquid colourless. The brown solid is soluble in dilute acids, giving a yellow solution, but it was not found possible to regenerate the pigment from it.

Fusion with sodium showed the presence of nitrogen in the pigment. When heated with zinc dust, vapours are evolved which impart a red colour to a pine-wood shaving moistened with hydrochloric acid, indicating the presence of pyrrol derivatives. The pigment is adsorbed by filter-paper, etc.

The pigment was first obtained by treatment of the fæces of certain hospital patients, and a pathological origin was suspected. Later it was found possible to isolate the pigment from the fæces of normal persons, and dietary influences were ruled out. Attempts to isolate the pigment from the fæces of infants, and of rabbits on normal and on milk diets were not successful.

Constitution of the Pigment.

Nencki and Sieber (1882), Garrod and Hopkins (1896) and Homer (1915) describe urorosein in urine which possesses the following properties in common with this pigment: similar absorption spectrum, similar slow development from its chromogen when treated with mineral acids, avid extraction by amyl alcohol from aqueous solution, insolubility in ether and chloroform, destruction by alkalies, but regeneration on *prompt* acidification. The pigment is thus identical with or closely similar to urorosein obtained from natural sources. It remains to point out the close similarity in properties and preparation to the synthetic products skatole red, indole red, etc. Fischer (1886) and Freund, and Lebach (1905) prepared dyes of the diphenylmethane type from indole and skatole with properties similar to those of the natural pigments. Homer (1913, 1915) and Fearon (1920) prepared red pigments of this kind by condensation of indole, skatole, tryptophane, etc., with aldehydes in the presence of condensing agents (*e.g.* hydrochloric acid) and oxidation of the chromogen obtained.

It is suggested, therefore, that the pigment described is a dye of the diphenylmethane type derived from some pyrrol derivative, *e.g.* skatole, in the fæces. The pyrrol compound condenses in the presence of alcoholic hydrochloric acid with a minute amount of aldehyde present as impurity in the alcohol or formed therefrom by oxidation, giving the chromogen, which when heated in the air is oxidized to the pigment. It was found that if skatole is heated on a water-bath with alcoholic hydrochloric acid containing a trace of acetaldehyde, a brilliant red pigment, destroyed by alkalies, was obtained.

SUMMARY.

The preparation, properties and probable constitution of a brilliant red pigment obtained from fæces are described.

The author's thanks are due to Dr. G. A. Harrison, who drew his attention to the formation of the pigment, for his interest and help.

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PRELIMINARY NOTE ON THE EXPERIMENTAL STUDY OF ENZOOTIC ENCEPHALO-MYELITIS (BORNA DISEASE).

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ENCEPHALO-MYELITIS has long been recognized as a specific infection of the horse under the name of Borna disease, but the ætiology has remained undetermined until recent years. The work of Moussu (1924), Zwick and Seifried (1924, 1926), Beck and Frohböse (1926) and Ernst and Hahn (1927) shows that the ætiological factor belongs to the group of filterable viruses, and is transmissible in series by intracerebral inoculation of the horse, sheep, rabbit, guinea-pig, rat and fowl; in addition, Beck and Frohböse, Moussu, and Miessner (1926) demonstrate that enzootic encephalo-myelitis of the sheep is produced by a virus which is analogous. Finally, Ernst and Hahn have succeeded in isolating from cases of enzootic encephalo-myelitis of cattle and from malignant catarrhal fever of the same species strains of virus which are very closely related to, if not identical with, those isolated previously from cases of Borna disease in the horse. These last authors are of the opinion also that deer become spontaneously infected with the same virus. The question of the comparison of different strains will be discussed later in our complete memoir. It is sufficient to say at present that we are led to believe from certain experimental data that, contrary to the opinion of Arndt (1926), and of Zwick, Seifried and Witte, the virus isolated by Moussu in France in various epizootics among horses and sheep is a more virulent strain of the same virus as that studied by German workers.

In the present preliminary note, the results are given of experiments undertaken with a strain of virus isolated by Zwick and his collaborators from horses, and with another isolated by Miessner from sheep.

The animal which is, apparently, most susceptible to infection experimentally with the pathogenic agent is the rabbit.

I.—*Certain properties of the virus.*—(1) After centrifugalization for 5 or even 15 minutes at a speed of 5400 revs. per min. the superficial layers are still found to be virulent. (2) The virus is sensitive to the action of ultra-violet rays; after 5 minutes' exposure in quartz flasks the virus is rendered inactive.

II.—*Experimental study in the rabbit.*—(1) First of all it should be

mentioned that all our experiments up to the present go to prove that the two strains studied, *i. e.* the strain isolated from horses (Zwick) and the strain isolated from sheep (Miessner), are identical. *Cross-immunity* experiments show that a rabbit immunized by intracerebral inoculation against the first of these two strains resists some thousand infective doses of the second strain inoculated by the same route. (2) Over 200 rabbits have been infected during the course of our experiments. The symptomatology of the experimental disease in the rabbit is characteristic of an encephalo-myelitis, which may finally result in general paralysis.

The period of incubation and the duration of the disease varies according to the individual susceptibility. The weight, that is to say, the age of the animal, appears to be the sole factor, having a marked influence upon such variation in receptivity. The average time elapsing between the date of infection by inoculation intracerebrally and the subsequent death of the animal in the case of the strain of Zwick has been 28 days in rabbits weighing less than 1500 gm. (observations made on 25 rabbits), and 36 days in rabbits weighing more than 1500 gm. (observations made also on 25 rabbits).

In the case of the Miessner strain of virus in 12 rabbits infected by intracerebral inoculation, death supervened on an average in 30 days. A fuller description of the clinical symptoms as well as the table which furnished us with these figures will be given in detail in the complete memoir.

A feature of capital importance is the loss of weight which takes place in infected animals, which may be as much as half of the original weight of the animal inoculated.

The temperature of experimental animals is uninfluenced by infection with the virus, although it becomes subnormal during the period preceding death. The changes in the blood as demonstrated by counts are inconstant, but the leucocytic formula shows that the number of mononuclear lymphocytes is markedly increased during the course of the infection. (3) According to Zwick and Moussu, the introduction of a few drops of emulsion of virulent brain into the *sciatic nerve* fails to lead to successful infection of the rabbit. In our experiments we have succeeded, however, in producing the typical disease several times in animals infected by this route. The first symptom noticed was paralysis of the leg supplied by the nerve into which the virus had been introduced. Following upon this, the typical syndrome developed. Characteristic lesions were found in the brain, spinal cord, roots of the peripheral nerves, the spinal ganglia as well as in the nerve inoculated. The presence of the virus in the central nervous system of rabbits infected by intrasciatic inoculation was determined by inoculating fresh animals intracerebrally with emulsions of their brains and spinal cords. (4) *Intratesticular inoculation* gave results similar to those obtained by intrasciatic inoculation. It was shown also that the brain and spinal cord of animals so infected contained virus, since fresh animals inoculated intracerebrally with emulsions of the central nervous system (brain and spinal cord) died from encephalo-myelitis after showing the usual typical symptoms. (5) *Intra-ocular* inoculation (anterior chamber), *corneal* scarification and *intravenous* inoculation do not give constant results. (6) The introduction of a thick virulent emulsion into the *trachea* of rabbits gave negative results (6 animals in 2 series of experiments). (7) Animals infected

by scarification of the previously depilated and shaved *skin* did not develop the disease. However, when the precaution was taken of previously irritating the brain by inoculation of sterile saline intracerebrally, the virus, travelling probably by the intercostal nerves, reached the central nervous system and produced the characteristic disease.

Inoculation of saline into the spinal cord of rabbits previously infected by scarification of the skin did not lead to localization of the virus in the central nervous system.

III.—(1) Our findings with regard to the transmission of the disease to *guinea-pigs* are in accordance with the results obtained by the German school. The period of incubation in this species of animal is still more markedly variable than in the case of the rabbit after intracerebral inoculation. After infection by this route guinea-pigs died after showing typical symptoms in anything from 19 to 130 days (the longest period yet observed by us). With the object of having a more general idea of the susceptibility of guinea-pigs to the virus, we inoculated 45 guinea-pigs, which were divided into three lots of 15. The first lot received intracerebrally a virulent emulsion diluted 1/10, the second a dilution of 1/100, and the third a dilution of 1/1000. The results were paradoxical. During the first 110 days which followed infection, the animals inoculated with a dilution of 1/1000 furnished the largest number of positive takes, which suggests that the sensitiveness of the guinea-pigs to the virus of encephalo-myelitis is very variable. (2) Attempts at infecting guinea-pigs by intradermal inoculation of the virus into the metatarsal region, employing the technique used originally in foot and mouth disease by Waldmann and Pape, and in experimental herpes by Gildemeister and Herzberg, gave negative results.

IV.—The virus of Borna is definitely pathogenic for the *rat*. We have inoculated in all 28 rats intracerebrally. The period of incubation and the symptomatology are exactly as in the guinea-pig. A fact worthy of special note, however, is that *old rats are much more susceptible than young ones*. In general, small animals of this species have resisted infection in our experience, while old animals have developed the typical disease.

V.—Mice infected by intracerebral inoculation may present symptoms characteristic of the disease ending in death, but they are more resistant to infection than guinea-pigs and rats. In this case also the older animals appeared more sensitive to the pathogenic action of the virus. Death took place 37, 52, 81, 125 days after infection respectively in our experiments.

VI.—Virus passaged through guinea-pigs or rats still retained its virulence for the rabbit. This is in accordance with the findings of Zwick. A fact of special interest, however, is that our rabbits inoculated with virus after passage through rats or guinea-pigs developed the disease after a shorter period of incubation (death in 19 to 21 days). These rabbits exhibited lesions which were excessively intense and characteristic in type.

VII.—(1) We have succeeded in conferring enzootic encephalo-myelitis to *monkeys*. A *Macacus rhesus* weighing 3800 gm. was inoculated intracerebrally with a virus of rabbit passage. The first symptoms of the disease developed 57 days afterwards. Paresis and paralysis supervened, and the animal died 16 days after the onset of symptoms, *i. e.* 73 days after infection. The

symptomatology was that of a typical poliomyelitic infection with an ascending paralysis (type Landry).

Rabbits were inoculated intracerebrally with emulsions from the various organs of the monkey with the object of determining the absence or presence of virus. The virus could only be found in those organs derived from the *ectoderm* (cerebrum, medulla, spinal cord, adrenal* and parotid*).

(2) The virus can be transmitted from monkey to monkey by intracerebral inoculation.

(3) Cross-immunity experiments with the virus of poliomyelitis are being carried out.

VIII.—The *ferret* appears refractory to intracerebral infection with Borna virus.

IX.—*Pathological histology of experimental enzootic encephalo-myelitis.*—(1) *Rabbit.*—Up to the present the various authors have confined their attention strictly to the central nervous system (brain and spinal cord) in studying the pathological history of Borna.

The lesions described are mononuclear meningitis, perivascular and parenchymatous infiltration, always of a mononuclear type, with, in addition, the oxyphilic corpuscles in the large ganglion cells of the *cornu ammonis* described by Joest and Degen in the horse many years ago. We have studied minutely the alterations *throughout* the nervous system, and the conclusions resulting from our experiments, which will be published in full later, include the following :

In the rabbit, inoculation of the virus of Borna disease intracerebrally produces lesions of a typical encephalo-myelitis, to which are added profound changes of the spinal ganglia and peripheral nerves (*encephalo-myelitis, ganglio-radiculitis and a peripheral neuritis*). In the central nervous system as well as the spinal ganglia histological modifications are of two orders—*infiltrative* and *degenerative.*

The meningitis and the parenchymatous and perivascular infiltrations are produced always by mononuclear cells. A lesion which we have noted constantly is that termed by us "*satellitism*" of the neuron (surrounding of the neuron by neuroglial cells and infiltrating mononuclears). This last modification of the normal structure of the nerve-tissue is more marked in the medulla oblongata, mid-brain and the spinal ganglia, but may exist throughout the central nervous system.

Sometimes this "*satellitism*" may go so far as to constitute true *neurono-phagia.*

Various stages and forms of *degeneration* of the nerve-cells are also met with—oxychromasia, chromatolysis, karyolysis, vacuolization of the cytoplasm which may show a denticulated border.

In the spinal cord, apart from the infiltrative lesions which predominate in the posterior horns, degenerative lesions of the nerve-cells may also be seen in the anterior horns. *Neuronophagia* is sometimes met with in the latter region.

In the *paravertebral ganglia* neuronophagia is much more frequent than in

* It is known that the medullary zone of the adrenal is derived from the ectoderm and that the parotid contains nerve-cells.

the central nervous system proper, and is comparable to that seen in experimental poliomyelitis of monkeys (disease of Heine-Medin).

The lesion of the *nerve-roots* and those of the *descending peripheral nerves* (sciatic nerve of animals inoculated into the brain) are produced by mononuclear cells, and present themselves as *interstitial* and *perivascular* infiltrations.

It is interesting to associate this peripheral neuritis obtained after central infection with the virus of Borna with the pathological process met with in zona zoster, "l'herpes recidivant," and perhaps also so-called "contagious sciatica," of which the ætiology is still unknown. It is possible that these latter conditions are external, secondary and descending manifestations of the respective viruses (which probably belong to the same group as the virus of Borna). The viruses concerned possibly descend from the central nervous system to the peripheral nerves centrifugally.

The possibility of centripetal ascending infection in these diseases must, however, not be excluded.

The corpuscles of Joest-Degen, intranuclear oxyphilic formations, appear to result from a condensation of the chromatin, which has become oxyphilic, possibly around the infectious agent. The cells which contain them present a marked rarification of the karyoplasm. It should be remarked that apart from this chromatic rarification, the cells with these corpuscles preserve their morphological and tinctorial integrity. Such corpuscles are never met with in degenerated cells or those undergoing degeneration. In rabies the same thing has been recorded. Negri bodies are seen only in cells which otherwise conserve their normal aspect.

We have found the corpuscles of Joest-Degen not only in the large ganglion cells of the *cornu ammonis*, but also in the pyramidal cells of the cerebrum, in the medulla oblongata, in the anterior horns of the spinal cord, and especially in the nerve-cells of the spinal ganglia.

It is perhaps worthy of note also that in the large ganglion cells of the *cornu ammonis* we have observed certain formations resembling so-called "cysts" in the cytoplasm in about 5% of cases. Their staining properties show that they are the degenerated nuclei of infiltrating mononuclear cells which have succeeded in penetrating the neuron.

(2) In the *guinea-pig* and the *rat* the alterations of the nervous system are on the whole comparable to those mentioned summarily in the case of the rabbit; the nerve-roots as well as the spinal ganglia show the same lesions as there described.

The sciatic nerve of infected guinea-pigs shows the same lesions as in the rabbit.

The corpuscles of the type described by Joest and Degen have been seen in the brain, spinal cord, and more especially in the spinal ganglia of infected guinea-pigs. These corpuscles also were found in the brain of rats.

(3) The lesions of the brain of the *mouse* infected by the intra-cerebral route have the same characters as in the aforementioned animals. Intranuclear corpuscles have also been demonstrated in the ganglion cells of the *cornu ammonis* of mice.

(4) In the *monkey* the whole nervous system is infected by lesions characteristic of an *encephalo-myelitis* with a *ganglio-radiculitis* and superadded *peripheral neuritis*.

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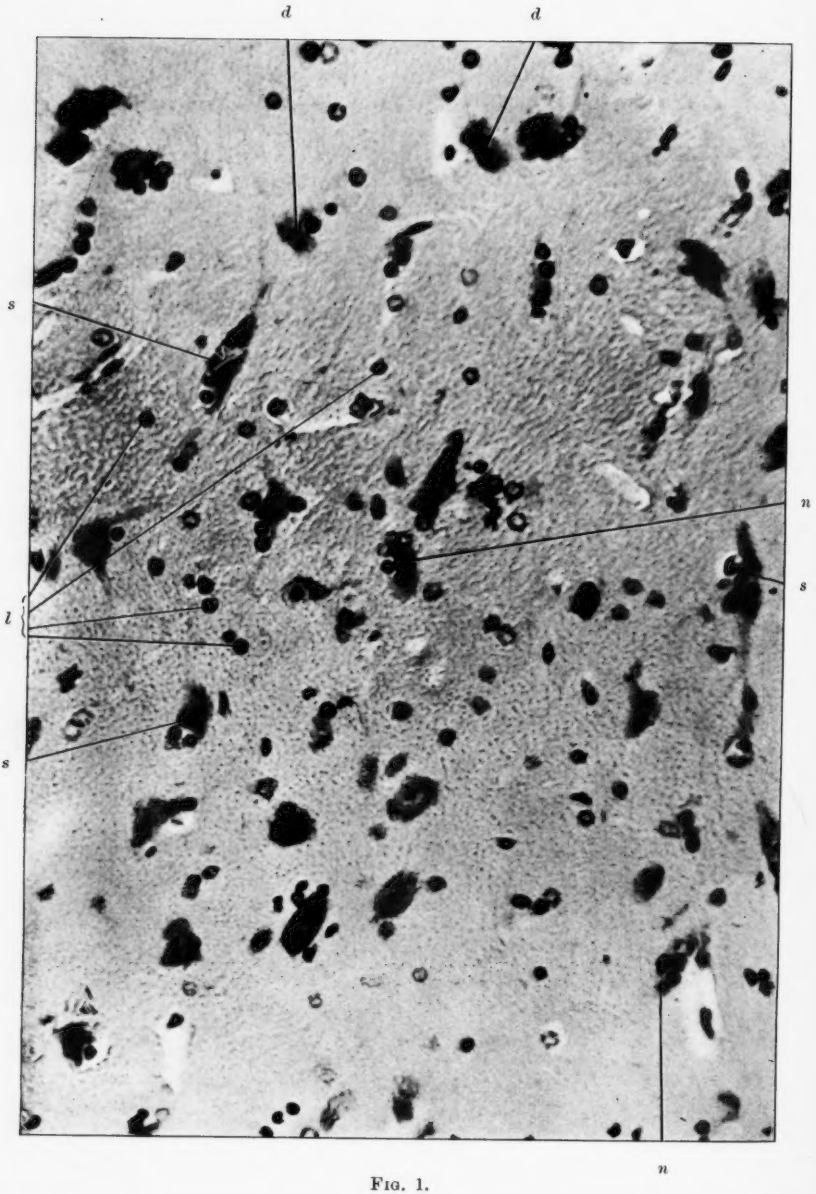
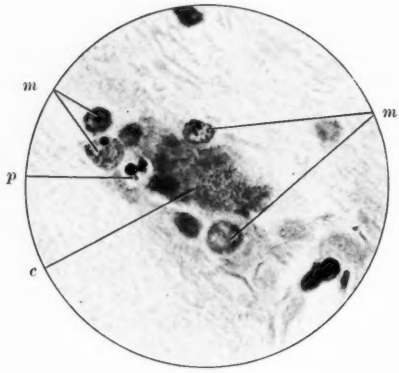


FIG. 1.

Nicolau and Galloway.



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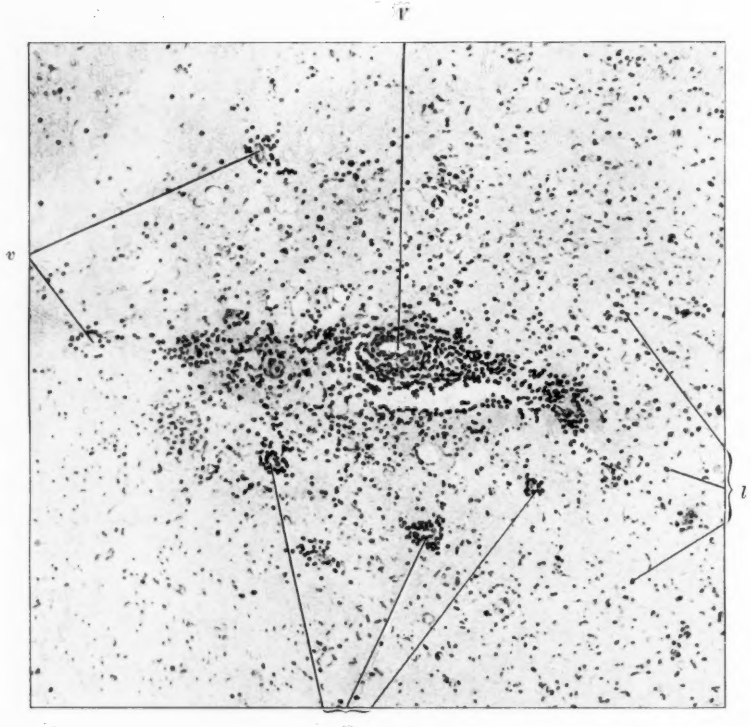

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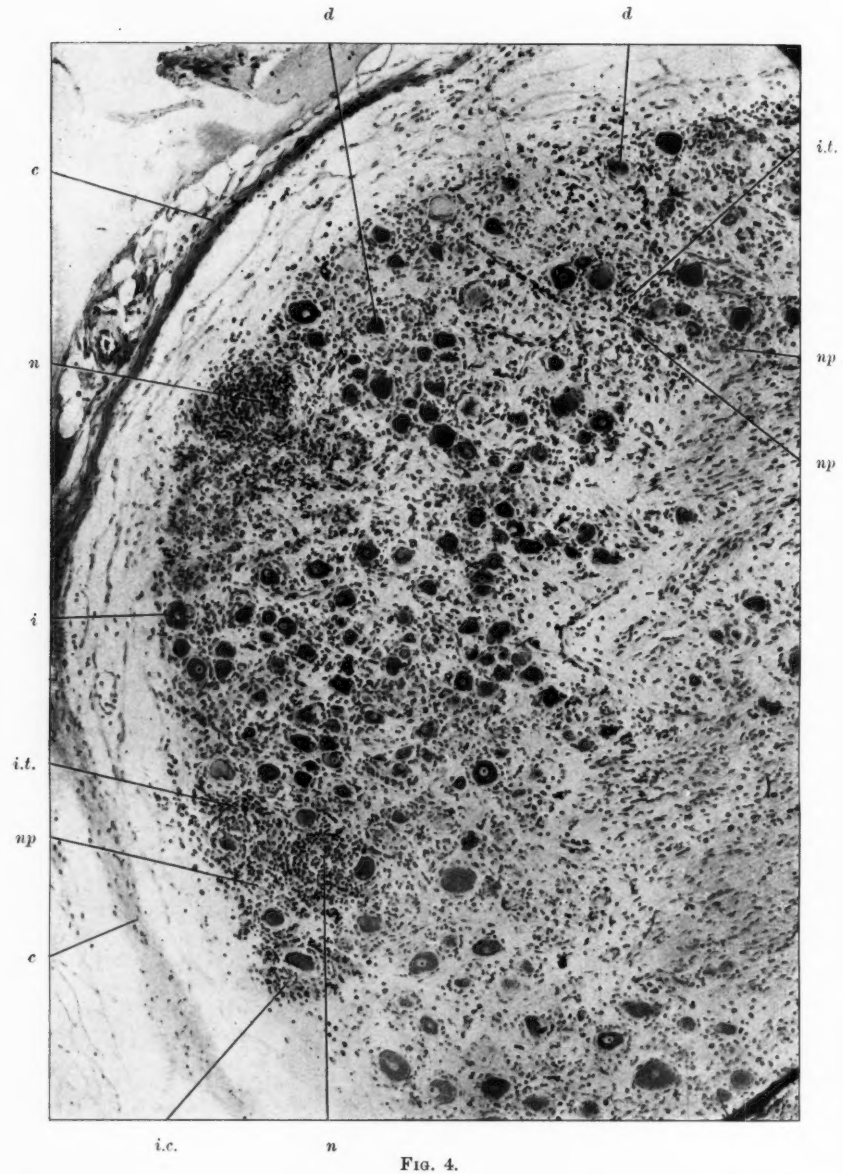

FIG. 4.

Nicolau and Galloway.

Neuronophagia is met with most frequently in the spinal ganglia. The details of all these lesions will be given in our monograph.

We have found typical oxyphilic corpuscles in nearly all sites of the nervous system (frontal, parietal and occipital lobes, hippocampus, optic thalamus, *cornu ammonis*, pons, medulla, cerebellum, cervical, thoracic and sacro-caudal parts of the spinal cord), but the finest and most frequent have been found in the spinal ganglia.

X.—(1) One can confer a durable immunity upon rabbits by intra-cerebral inoculation of a suitably attenuated virus. According to our experiments such immunity may last at least 160 days.

(2) The strain of encephalo-myelitis virus isolated from the horse infected with Borna (Zwick strain) suitably attenuated renders rabbits resistant to the intra-cerebral inoculation with the virus isolated from the brain of sheep (Miessner strain).

(3) Rabbits hyper-immunized against herpes by intra-cerebral inoculation are still susceptible to infection with the virus of Borna introduced by the same route.

We beg to thank the Medical Research Council for putting at our disposal all the material necessary for carrying out this research, and especially Capt. S. R. Douglas, Director of the Department of Pathology, for enabling us to bring this work to a successful conclusion. We also thank Prof. Zwick and Prof. Miessner, who kindly put their viruses at our disposal

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DESCRIPTION OF PLATES. (MICROPHOTOGRAPHS).

Fig. 1.—Rabbit infected by intra-cerebral inoculation. Section in the region of the pons showing—(*d*) degenerated nerve-cells; (*s*) "satellitism" with commencing neuronophagia; (*n*) neuronophagia; (*l*) lymphocytes in the parenchyma. × 230 (photo enlarged 2 diameters).

Fig. 2.—Rabbit infected by intra-cerebral inoculation. Nerve-cell showing neuronophagia in medulla oblongata. (*c*) Degenerated cytoplasm; (*p*) polynuclear penetrating neuron; (*m*) monocytes. × 1000.

Fig. 3.—Monkey infected by intra-cerebral inoculation. Transverse section from sciatic nerve after leaving obturator foramen. (*V*) Large vessel with perivascular infiltration; (*v*) small vessels showing same phenomena; (*l*) lymphocytes (interstitial infiltration). × 55 (photo enlarged 2½ diameters).

Fig. 4.—Monkey infected by intra-cerebral inoculation. Section of spinal ganglion in lumbar region of spinal cord showing the profound changes which predominate the peripheral parts of the nervous system. (*i*) Intra-nuclear corpuscle (Joest-Degen) with surrounding halo; (*n*) nodule of monocytes replacing destroyed neurons; (*d*) degenerated ganglion cells; (*np*) neuronophagia; (*i.c.*) Pericellular infiltration; (*i.t.*) interstitial infiltration; (*c*) capsule of ganglion. × 36 (photo enlarged 2½ diameters).